

***IN VIVO* IMMUNE RESPONSES IN CATTLE  
FOLLOWING IMMUNISATION WITH  
*THEILERIA ANNULATA* CELL LINES**

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**1996**



## ABSTRACT

*Theileria annulata*, a protozoan parasite, causes a lymphoproliferative disease of cattle known as tropical theileriosis. This disease is a serious constraint to the improvement of indigenous cattle using crossbreeding in developing countries and is usually fatal in exotic and cross-bred animals if not treated. *T. annulata* infected tissue culture cell lines are used as a vaccine in most parts of the affected world. The lymph node draining the site of immunisation is believed to be the site for immune responses but the mechanism of responses in the draining lymph node (DLN) and afferent lymph was unknown when this work began. This thesis set out to investigate responses in the DLN and afferent lymph after cell line immunisation by immunohistological, cytokine and pseudoafferent cannulation studies.

Immune responses in the DLN were examined by immunohistological and reverse transcription polymerase chain reaction (RT-PCR of cytokines) methods. Initial activation of T cells was observed in the paracortex (the normal anatomical site for activation) by day 4 after immunisation. Primary follicles (PF) were observed by day 4, developed germinal centres (GC) by day 12 which increased in size and number by day 16. A high, 1:10240, schizont serum antibody titer was recorded on day 16. A few T cells (T cells are critically important for GC formation) were observed in the PF. These increased by day 16 when well developed GCs were observed. Similarly, a few proliferating cells were noticed in PF by day 4. These had increased by day 9 and were numerous by day 16, by which time PF had become large GCs. VPM30<sup>+</sup> cells were found in the light zone of GCs on day 16 only. This molecule is expressed on differentiated B cells in the GC. Schizonts were observed in CD11b<sup>+</sup> cells, this molecule is expressed upon monocyte/macrophages and granulocytes. Proliferating cells were observed by day 4 in the paracortex and by day 9 & 16 in the medulla.

Cytokine profiles of the DLN were assessed by RT-PCR using primers for IL-2, IL-2R, IL-4 and IFN $\gamma$ . Nodes of naive animals expressed mRNA for IL-2, IL-2R, IL-4 and IFN $\gamma$ . By day 4 after immunisation, weak mRNA for IL-2, very strong mRNA for IL-2R were detected, mRNA for IL-4 was undetectable whereas level of mRNA for IFN $\gamma$  increased. The mRNAs IL-4 as well as IFN $\gamma$  were undetectable on day 12; these had reappeared by day 16. The mRNAs for IL-2 and IL-2R were observed until day 16 following immunisation.

Transfer of the schizont from immunising to recipient's cells is considered to be a prerequisite for the generation of immunity, but the site of this transfer was unknown when the work began. Cannulation studies were undertaken to investigate parasite transfer and T cell activation in the afferent lymph. A novel method of cannulation was devised to collect afferent lymph. Fluorescent dye stained, *T. annulata* infected cells drained into the cannula within 15-30 minutes after immunisation. The *T. annulata* infected cell lines reisolated from the afferent lymph were only of donor origin. It is hypothesised that the DLN is the site for parasite transfer from donor to the recipient cells. An increase in CD2<sup>+</sup> and CD8<sup>+</sup> cells was observed. Activation of T cells was observed with an increase in expression of MHC class II on CD8<sup>+</sup> cells.

A decrease in  $\gamma\delta$ T cells was observed which was associated with an increase in CD2<sup>+</sup> cells. Only memory T cells as analysed by phenotype were observed before immunisation whereas naive T cells were also observed after immunisation in the afferent lymph. Afferent lymph cells proliferated in *in-vitro* assays in response to the immunising as well as to the autologous cell line. These lymph cells were cytotoxic to the allogeneic immunising cell line as well as to the autologous cell line as assessed by cytotoxic assays.

Natural killer like cells (NK) (CD2<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> CD3<sup>-</sup>) with activated phenotype (CD45RO<sup>+</sup>) were observed in the afferent lymph after immunisation. The levels of IFN $\gamma$  recorded on day 0 (30pg/ml) increased to 900pg/ml between days 4-6 after immunisation. A weak non-MHC restricted cytotoxicity was observed during the first week of immunisation.

Results presented in this thesis showed that T cell activation and formation of GCs occurred in the DLN following cell line immunisation. Cytokine studies showed production of IL-2, IL-2R, IFN $\gamma$  followed by IL-4. This was in complete contrast to studies by other workers on lethal sporozoite infections where GCs were lost and only IFN $\gamma$  was detected. Cannulation studies indicated DLN to be the site of parasite transfer and showed: the circulation of naive T cells through the afferent lymphatics, the site of immunisation as the initial T cell activation site and NK-like activity at the site of immunisation.

### **Declaration**

I hereby declare that the work presented in this thesis is my own, except where stated in the text. The work has not been submitted in any previous application for a degree.

Parveen Goel



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## ABBREVIATIONS

Abbreviation	Full form
ACD	acid citrate dextrose
ALC	afferent lymph cells
APC	antigen presenting cells
BCG	Bacille Calmette Guerin
BoLA	bovine leucocyte antigen
BPA	bovine plasma albumin
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
Con A	concanavalin A
CTL	cytotoxic T lymphocytes
CTLp	cytotoxic T lymphocyte precursors
DLC	differential leucocyte count
DLN	draining lymph node
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTH	delayed type of hypersensitivity
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ELL	efferent lymph lymphocyte
FACS	fluorescent activated cell sorter
Fc	fragment crystalline
FCS	foetal calf serum
FSC	forward scatter
GAM	goat anti mouse
GC	germinal centre
GUTS	ground up tick supernates
HBSS	Hank's balanced salt solution
HEV	high endothelial venules
HIV	human immunodeficiency virus
hr	hour
IEF	Isoelectric focusing
i.u.	international units
IFAT	indirect fluorescent antibody test
IFN	interferon
IFN $\gamma$	interferon gamma
Ig	immunoglobulin
IL	interleukin
IL-2R	interleukin-2 receptor
kD	kilodalton
LCA	leucocyte common antigen
LFA	leucocyte function antigen



log	logarithmic
LPS	lipopolysaccharide
mAb	monoclonal antibody
mCi	millicurie
MEM	minimum essential medium
MHC	major histocompatibility complex
min.	minute
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
mM	millimole
mw.	molecular weight
mRNA	messenger ribonucleic acid
mV	millivolts
NK	natural killer like
NGS	normal goat serum
NMS	normal mouse serum
OD	optical density
PBM	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
PE	phycoerythrin
PF	primary follicle
PI	propidium iodide
per. comm.	personal communication
PG	prostaglandins
PMT	photomultiplier detector
R 1	region 1
RAB	rabbit anti bovine
RAM	rabbit anti mouse
RBCs	red blood cells
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
S.G.	specific gravity
SIV	simian immunodeficiency virus
spp.	species
SSC	side scatter
t.e.	tick equivalent
temp.	temperature
TC	tissue culture
TCGF	tissue culture growth factor
TCR	T cell receptor
TEC	total erythrocyte count
T <sub>HI</sub>	T helper cells-type 1
TLC	total leucocyte count

TNF	tumour necrosis factor
VCAM	vascular cell adhesion molecule
WC	workshop clusters

## ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Dr. Roger L. Spooner, for excellent surgery for lymphatic cannulations and providing superb facilities in the laboratory.

My sincere thanks to my university supervisor, Dr. Patricia M. Preston for her encouragement and support and her constructive criticisms during the writing up of the thesis.

I would like to thank Prof. Duncan Brown and the staff at CTVM for the ready supply of tick material.

My sincere thanks to Dr. Barry Thorp for his help in histology; Marjorie Ritchie for surgical facilities and post-operative care of the experimental animals; Susan Craigmile for her help in RT-PCR; Dr. JDM Campbell for immunohistology and Dr. Anil Nichani for his support in flow cytometry. I also extend my thanks to farm staff: John, Harry Bowran, Douglas, Bill Seawright and George.

Help and support of colleagues in the laboratory: Dr. Liz Glass, Dr. George Russell, Dr. Suminder Sawhney, Dr. AP Usha, Bob Oliver, Aileen Morgan and Paula Miller is thankfully acknowledged.

I am most grateful to Dr. John Hopkins, University of Edinburgh, Dr. Chris Howard, IAH Compton, Prof. Johannes Gerdes and ILRI, Kenya for the gift of mAb. and Dr. Garry Entrican for the gift of bovine recombinant interferon-gamma.

My thanks to Norman Russell, Roddy Field and Elliot Armstrong of photography and graphics for all their efforts.

I am indebted to the Commonwealth Scholarship Commission in the United Kingdom for providing financial support and to my employer, the CCS Haryana Agricultural University, Hisar, India for providing study leave to enable me to undertake this study.

Finally, my gratitude to my wife Seema, for her patience and encouragement during this work and my son Harshit for his love.

# **CHAPTER 1**

## **INTRODUCTION**

Between 1960 and 1990 the total global human population increased by 75 percent from 3.1 billion to 5.4 billion with populations in developing countries increasing by 97 percent from 2.097 billion to 4.138 billion (FAO, 1992). During the same period the food grain production per capita increased from 310 to 375kg overall but from only 190 to 260kg in developing countries (Pinstrup-Andersen, 1994). 800 million people are now suffering from malnutrition and hunger (FAO, 1993). The world's population is expected to increase still further from 5.4 billion in 1990 to about 7.2 billion in 2010. This increase will have major detrimental effects on the per capita availability of food.

Livestock production is a major component of the agricultural policies of developing countries and the sale of livestock and their products provides direct cash income to farmers. In 1990, the consumption of meat and milk in developing countries was only 22 and 18 percent per capita of that in developed countries. To bridge this gap in consumption, it will be essential to adopt measures to increase food production from livestock. Although cattle are considered to be the most important animal source for food and are numerous in the tropics, their productivity is extremely low in tropical countries. For example in Northern India, which is considered as the milk bowl of India, the average milk production is about 6-7 litres per indigenous cow whereas milk production is far lower in rest of the Subcontinent. In the tropics, attempts to increase cattle production are hampered by climate, nutrition, management, low producing genetic traits and last, but not least, disease.

Breeding programmes in which indigenous cattle were crossed with exotic European breeds were initiated some 30 years ago but were severely hampered by tick borne-protozoan diseases as both the exotic cattle and their crosses are highly susceptible

to these diseases (Gill *et al*, 1980). The bovine theilerioses are among the most important of these diseases (Uilenberg *et al*, 1993). Theilerioses include the diseases caused by *Theileria annulata* (Dschunkowsky & Luhs, 1904) and *Theileria parva* (Koch, 1906). In 1982, it was estimated that over 250 million cattle were at risk from *T. annulata* infection alone (Robinson, 1982).

Tropical theileriosis places an enormous burden on the economy of developing countries. For example in India, out of 44,000 adult crossbred cattle in one district, 3,800 clinical cases of the disease were found in the year 1988-89 (Singh, 1991). In addition to the cost of treatment, control of the tick by the use of acaricides is very expensive. In addition to this, production losses due to mortality are enormous. In a recent study, losses of over \$15 million/ year were identified in Morocco alone (E. Flach per. comm.).

Transmitted by ticks of the genus *Hyalomma* to susceptible cattle, *T. annulata* sporozoites invade leucocytes and develop into intracellular macroschizonts in the lymph node draining the inoculation site. The macroschizont-infected cells are induced to divide rapidly. The parasite then differentiates via the microschizont stage into merozoites. Rupture of the host cells accompanies release of merozoites which invade erythrocytes to form piroplasms. Piroplasms and macroschizonts are both thought to be involved in the pathogenesis of tropical theileriosis (Neitz, 1957; Sharma & Gautam, 1971; Hooshmand-Rad, 1976; Uilenberg, 1981, Preston *et al*, 1992a; Nichani, 1995).

Macroschizont-infected cell lines can be initiated *in vitro* and these cell lines can be attenuated by prolonged culture as reviewed by Brown, 1983. The attenuated cell

lines used as vaccines have been developed by continuous passage in culture and tested for immunogenicity by inoculation into susceptible calves at different passage levels. In most instances, 2-3 years of continuous *in vitro* culture have been required for attenuation. Although such cell line vaccines are now widely used, very little is known about the processes underlying attenuation. Cell lines attenuated by prolonged *in vitro* culture have been used successfully as vaccines in many countries including Iraq (Hooshmand-Rad, 1973), Iran (Hashemi-Fesharki & Shad-Del, 1973; Hashemi-Fesharki, 1988), China (Gansu Provincial Institute of Veterinary Medicine, 1975; Wenshun *et al*, 1982), Russia (Stepanova *et al*, 1977), Israel (Pipano, 1978; 1981), Turkey (Ozkoc & Pipano, 1981), India (Singh, 1990; Shukla & Sharma, 1991), and Morocco (Ouhelli, 1991).

Although the transfer of the parasite from the macroschizont-infected cell lines used as vaccines to the cells of recipients is considered a prerequisite for the development of immunity, the site and mechanism of transfer are unknown. Knowledge of the immune responses of cattle to *T. annulata* infected cell lines is still limited although macroschizont-infected cell lines are known to stimulate humoral responses (Shukla & Sharma, 1989; 1991; Kachani, 1992a,b) as well as cell mediated immunity (Preston *et al*, 1983; Innes *et al*, 1989a; Nichani, 1995). There are two phases of CMI, initially against the MHC antigens of the cell line and later to the parasitised cells of the autologous cell line (Preston *et al*, 1983; Innes *et al*, 1989a). Recently, both these phases of immune response were observed in the efferent lymph of the node draining the site of immunisation (Nichani, 1995).

However, nothing is known about the activation of T cells in the draining lymph node (DLN) and the afferent lymph which drains the site of immunisation with *T.*

*annulata* infected cells. This thesis investigated the role of macroschizont-infected cell lines in stimulating host responses in the afferent lymph and draining lymph node. The aim being to begin to understand the type of responses which cattle produce when immunised with cell line vaccines.



## **CHAPTER 2**

### **LITERATURE REVIEW**

## 2.1 BOVINE THEILERIOSES

The diseases caused by *T. annulata* and *T. parva* are among the most serious constraints to livestock development and production in developing countries (Irvin & Morrison, 1987). Since this thesis is concerned with immunisation of cattle with *T. annulata* infected cell lines, the review will concentrate on the *T. annulata* parasite and *in vitro* derived macroschizont-infected cell lines. Reference to the related parasite *T. parva* will be made where appropriate. Bovine lymphoid system, leucocyte markers and role of cytokines have also been reviewed as this study investigated various leucocyte subpopulations and cytokines involved in the generation of immune responses in the lymphoid organs.

### 2.1.1 Background and classification

*T. annulata* and *T. parva*, both parasites belonging to the genus *Theileria*, were first recognised in 1904 and 1906 respectively (Dschunkowsky & Luhs, 1904; Koch, 1906). Both these species originally belonged to the genus called *Piroplasma* but were later assigned to a new genus *Theileria* (Bettencourt *et al*, 1907). A total of 39 *Theileria* species have been identified (Levine, 1988). Various *Theileria* species have been reviewed extensively (Sergent *et al.*, 1945; Neitz, 1957; Barnett, 1968; Barnett, 1977; Purnell, 1977; Irvin & Morrison, 1987). Only *T. annulata* and *T. parva* have been studied in detail. On the basis of a recent classification of protozoa (Levine, 1988), the currently accepted classification of *Theileria* is illustrated in the following table (from Irvin, 1987 and Norval *et al*, 1992).

Kingdom	Protista
Sub Kingdom	Protozoa
Phylum	Apicomplexa
Class	Sporozoa
Sub Class	Piroplasmia
Order	Piroplasmida
Family	Theileriidae
Genus	Theileria

### 2.1.2 Distribution and transmission

*T. annulata*, the cause of bovine tropical theileriosis, was originally identified in southern Russia by Dschunkowsky and Luhs (1904). Today bovine tropical theileriosis is recognised as a widespread disease occurring mainly in tropical and subtropical regions from Portugal, Spain and Morocco in the west, through the Mediterranean coast of Europe and North Africa, southwards into the Sudan and Eritrea and eastward into south-east Europe, the Near East and Middle East, southern Russia and Siberia and across the Indian subcontinent through to China and the Far East (Purnell, 1978, Robinson, 1982). *T. parva*, in contrast, is limited to eastern, central and southern Africa (Norval *et al*, 1992; Uilenberg *et al*, 1993). Sudan is the only country where both the parasites have been identified (Norval *et al*, 1992).

The different distribution of the two species (Fig. 2.1) is assumed to be because of the difference in their vector ticks. *Theileria* parasites are transmitted trans-stadially by two and three host ticks i.e. from larva to nymph or nymph to adult (Robinson, 1982). *T. annulata* is exclusively transmitted by ticks of the genus *Hyalomma*. The three host tick *H. anatolicum anatolicum* is the vector from northwest Africa eastward to and including India. It is replaced by another three host tick *H. lusitanicum* in the southwest Mediterranean region and Spain (Barnett, 1977). The two host tick *H. detritum* is a common vector for tropical theileriosis from Manchuria through China and the former southern Soviet Union, south eastern Europe, the middle East and the Mediterranean littoral of Africa (Norval *et al*, 1992). Although *T. parva* is transmitted by *Rhipicephalus* and *Hyalomma* ticks, *Rhipicephalus appendiculatus* is the most common vector (Purnell, 1978; Norval *et al*, 1992).

Transmission of the sporozoites from tick to cattle occurs two to four days after

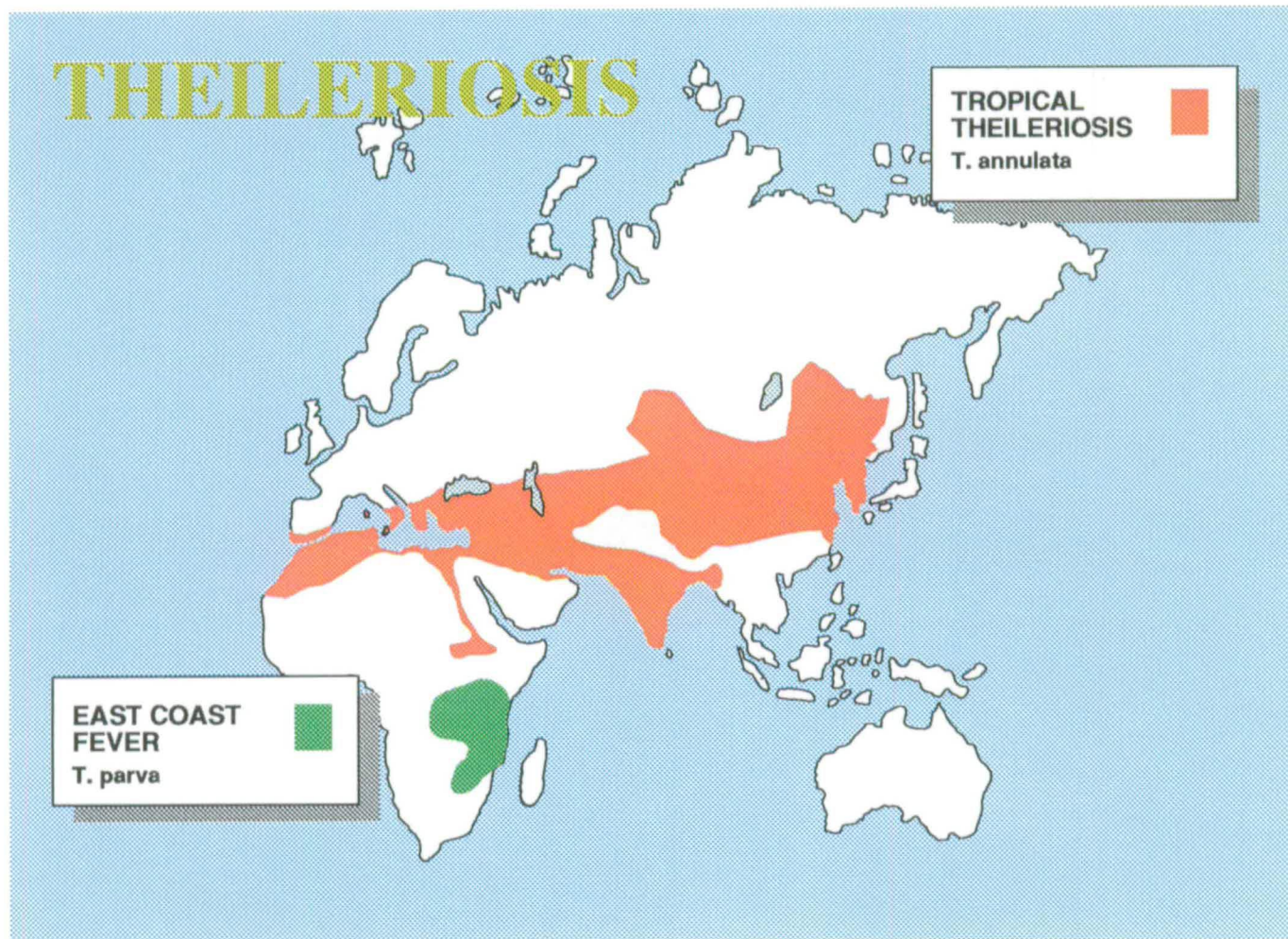


Fig. 2.1: Distribution of *Theileria annulata* and *Theileria parva*.

attachment of the tick on a susceptible host and the stimulus of a blood meal is required to induce maturation of the parasite in the tick salivary gland (Purnell *et al*, 1973; Samish & Pipano, 1978; Singh *et al*, 1979). However, increasing the temperature and relative humidity also leads to maturation of the parasite in the salivary glands in the absence of blood meal (Samish, 1977; Young *et al*, 1979). This suggests that maturation of parasite in the salivary glands of ticks might occur earlier under natural conditions in hot areas so that ticks could transmit the infection as soon as they attach to a susceptible host. Engorgement of an infected tick on a clean susceptible host leads to the following instar being clear of infection.

### **2.1.3 Host and susceptibility**

Although sporozoites of *T. annulata* have been shown *in vitro* to invade cells from cattle, buffalo, goat and sheep, (Steuber *et al*, 1986), field cases are found only in cattle, buffalo, yak and their cross breeds (Barnett, 1977; Wenshun, 1994). Exotic *Bos taurus* breeds of cattle and their crosses are highly susceptible to *T. annulata* whereas local Zebu and taurine breeds reared in the endemic areas are usually resistant (Singh, 1990; Hashemi-Fesharki, 1991; Flach & Ouhelli, 1992). Young *Bos indicus* calves also suffer from the disease. Adult *Bos indicus* and indigenous taurine breeds are thought to be relatively resistant. In China, yak crossbreeds with ox are highly susceptible (Wenshun, 1994). Recently, Preston *et al*, (1992a) showed that sahiwal x *Bos taurus* crossbred calves were relatively more resistant to tropical theileriosis than taurine calves. Similarly, local breeds were shown to be more resistant than exotic taurine breeds in East Coast fever endemic areas (Guilbride & Opwata, 1963). Moll *et al*, (1986) demonstrated that few Zebu calves died from *T. parva* infection in endemically stable areas although high numbers of improved taurine cattle would die if introduced to these areas.

Firm evidence for genetic control of resistance to tropical theileriosis or East Coast fever is lacking (Spooner & Brown, 1991). Local cattle from the resistant breed reared in disease free conditions were found to suffer more than imported cattle after challenge with *T. annulata* (Sergent *et al*, 1945). It is not clear whether the resistance observed in local breeds in endemic areas is because of genetic factors due to selection during the years cattle have been associated with the parasite or is because early exposure of young animals to the parasite makes them immune.

The pattern of occurrence of theileriosis in cattle of different ages varies between countries. In India, clinical theileriosis is a serious problem in new-born calves up to 8 weeks of age (Grewal *et al*, 1991; Nichani, Kumar, Goel & Sharma, unpublished observations). In Pakistan, young animals below nine months of age were reported to suffer with the worst affected animals being between 2-4 months of age (Hussain *et al*, 1991). However, in Morocco very young calves are less affected (Flach & Ouhelli, 1992). Similarly, in Egypt, animals of 1-2 years of age were reported to suffer (Tawfik, 1996). These differences could be due to the feeding pattern of different vector ticks. In areas where the disease is transmitted by two host ticks, only the adult stage of the tick is capable of transmitting the infection. Since the adult ticks feed preferentially on adult animals, young calves are less likely to be infected. In areas where the disease is transmitted by three host ticks, both nymph and adult ticks can transmit the disease. Since the immature ticks feed equally well on both adult and young animals, young animals are also infected.

#### **2.1.4 Life cycle**

The life cycle of the *Theileria* is illustrated in figure 2.2. The life cycle of *Theileria* has also been reviewed by Sergent *et al*, (1945), Neitz (1957), Schein (1975),

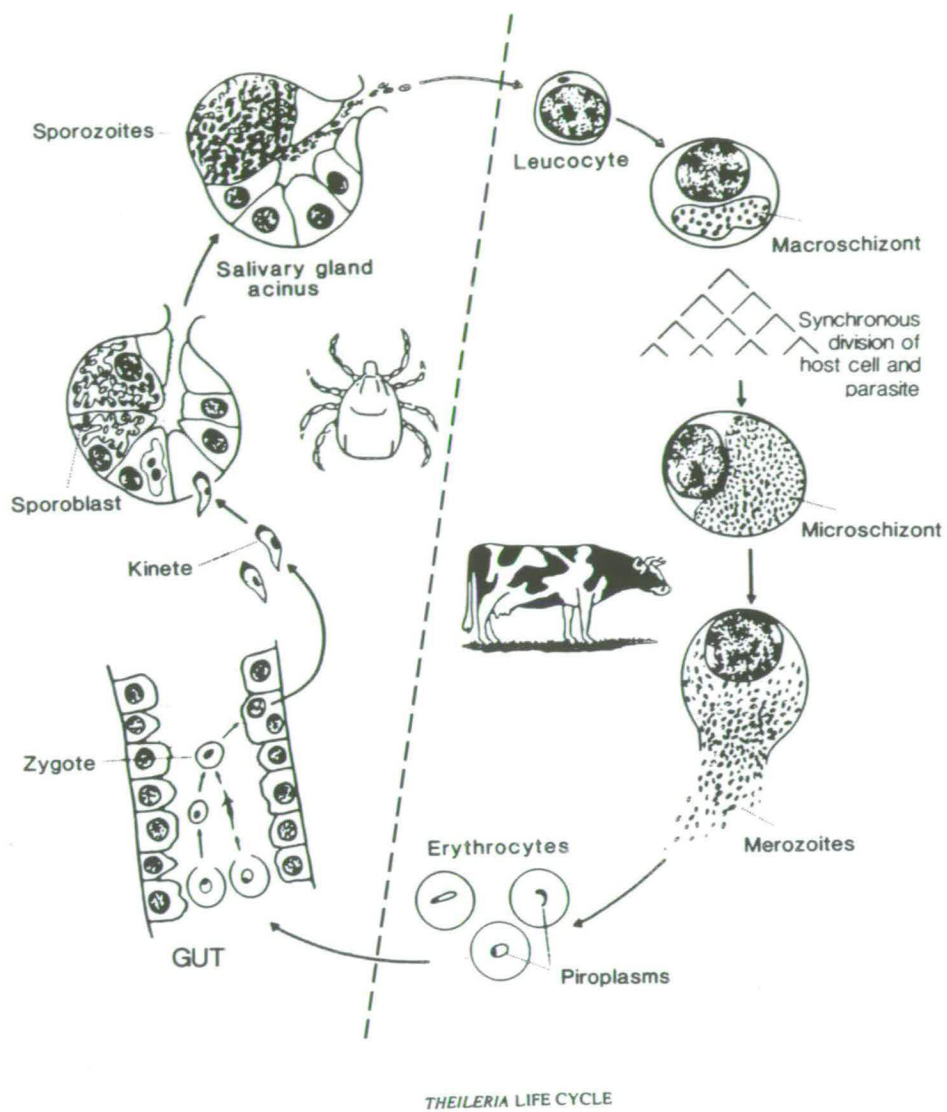


Fig. 2.2: Life cycle of *Theileria* sp.



Uilenberg (1981). The two main stages in the tick, gametogony and sporogony, have been described in detail by Cowdry & Ham (1932), Melhorn & Schein (1984) and Melhorn & Schein (1993).

#### **2.1.4.1 *Development within cattle***

Cattle are infected through inoculation of sporozoites by saliva of infected ticks. In *in vitro* studies on *T. annulata* (Jura *et al*, 1983a) and on *T. parva* (Shaw *et al*, 1991) sporozoites quickly invade cells (within 5-60 minutes). It is not known where the sporozoite encounters the bovine target cells for invasion *in vivo*. It could be in the mouthparts of the tick, the tick attachment lesion or the pool of cells and fluids that develops below this lesion (Walker & Fletcher, 1986), in the afferent lymph or in the lymph node draining the site of inoculation. No ligand has yet been identified for sporozoite-host cell recognition and invasion. However, a receptor-ligand driven process (Tait & Sacks, 1988) and invagination of the plasma membrane of the target cell in an active temperature dependent process has been postulated (Jura *et al*, 1983b).

After entry into the host cell, sporozoites develop into multi-nucleate trophozoites which after nuclear division develop into macroschizonts which appear 5-8 days after infection in the draining lymph node (DLN). In experimental conditions, the time at which macroschizont-infected cells are first detected is dependent upon the dose of sporozoites injected (Preston *et al*, 1992a). Macroschizont-infected cells leave the DLN, via efferent lymph, immediately after their first appearance in the DLN (Nichani, 1995). Infected with the macroschizont stage, host cells enter into division cycles and the parasite divides synchronously with the host cells leading to an increase in the number of macroschizont-infected cells (parasitosis) in the node.



Parasite replication occurs with DNA synthesis during the host G<sub>2</sub> phase and the parasite divide before the host metaphase (Jura *et al*, 1985). It apparently divides along with host cell spindle (Hulliger *et al*, 1964; Hulliger, 1965). Macroschizont-infected cells divide, *in vitro*, without any growth factor. Recent studies indicate that the infected cells express the Ki-67 nuclear proliferation antigen (Shayan *et al*, 1994). This antigen is expressed in cells outwith the G<sub>0</sub> phase of the cell cycle (Gerdes *et al*, 1984) and its expression shows that infected cells are stimulated to enter the cell cycle permanently. After several cycles of clonal expansion, macroschizonts differentiate into microschizonts and start producing merozoites *in vivo*.

Differentiation to merozoites occurs within the host cell by increased nuclear division and the generation of rhoptry and microneme organelles (Melhorn & Schein, 1984). The stimulus to form the merozoites *in vivo* is not known. Presumably, fever is responsible as merogony can be induced *in vitro* in *T. annulata* cell lines by raising the temperature of the culture to 41°C (Glascodine *et al*, 1990). This process results in significant changes in the antigenic profiles of the parasite, indicating that merogony is a major point of differentiation in the mammalian phase of the parasite life cycle and that new gene expression occurs at this stage (Glascodine *et al*, 1990). The number of theilerial particles per cell increased by a disruption in the synchrony between parasite growth and host cell division (Shiels *et al*, 1992). This development leads to rupture of host cell and release of merozoites.

Once released, free merozoites invade erythrocytes and develop into piroplasms which can be detected within 9 days after experimental infection. The mechanism of penetration of merozoites into erythrocytes is not known and is presumed to be similar to the entry of sporozoites into host cells (Norval *et al*, 1992). The piroplasms

undergo division within the erythrocytes by binary fission (Melhorn & Schein, 1984) or schizogony (Conrad *et al*, 1985). These erythrocytic parasites persist in extremely low levels throughout life in recovered animals (Sergent *et al*, 1945).

#### **2.1.4.2 Development within tick**

The sexual stages of *T. annulata* (Sergent *et al*, 1945; Neitz, 1957; Schein, 1975) and *T. parva* (Cowdry and Ham, 1932; Melhorn and Schein, 1984) develop within the vector tick. Upon ingestion, piroplasm-infected erythrocytes are lysed, within the gut of the tick, giving rise to sexual stages (gametes) which combine to form zygotes within 3 days (Schein, 1975). The zygotes enter the gut epithelial cells and develop into motile club shaped kinetes. The time taken for kinete formation and migration to the salivary gland is reported to be variable (7-20 days) and may depend upon the moult status of the tick (Schein & Friedhoff, 1978; Young *et al*, 1980; Reid & Bell, 1984). Kinetes develop in the cells of the type II or type III acini (Schein & Friedhoff, 1978) where they round up forming sporoblasts which can be detected one or two days after the tick finishes moulting. When the tick attaches to a new host and commences feeding, nuclear division in the sporoblasts (sporogony) produces infective uninuclear sporozoites which are released in the tick saliva and injected into the new host. About 50,000 sporozoites are estimated to develop in a single acinar cell. This amounts to an enormous inoculum from one feeding tick, when many acini of the salivary gland are infected (Melhorn & Schein, 1993). Feeding on nonsusceptible hosts (eg. rabbit) induces maturation but not transmission, this process can be used for obtaining sporozoites for *in vitro* use.

#### **2.1.5 Target leucocytes for invasion by sporozoites**

The preferred target cell for sporozoites of *T. annulata* and *T. parva* after inoculation

into an animal or *in vitro* has been a matter of debate for years mainly because of lack of suitable reagents for identifying populations of infected cells. With the development of monoclonal antibodies (mAbs) for bovine leucocyte populations (Lalor *et al*, 1986), much information became available to define potential targets for infection by sporozoites. *T. annulata* sporozoites were subsequently shown to infect and transform cells of the monocyte and macrophage lineage and B cells but not T cells, whilst *T. parva* sporozoites infected and transformed T cells and B cells but not monocytes/ macrophages (Spooner *et al*, 1989). Mammary gland macrophages were permissive to infection by *T. annulata* but only to a limited extent to infection by *T. parva* (Glass *et al*, 1989). Recent sorting experiments have shown *T. annulata* sporozoites to infect mature monocytes more readily, although immature monocytes were also easily infectable (Campbell *et al*, 1994a) where they lost the immature markers. Bovine cytotoxic T cell lines were very easily infected by *T. parva* sporozoites but not by *T. annulata* sporozoites (Innes *et al*, 1989c).

Monocyte/macrophages, which expressed the markers CD14/IL-A109 and the elastin receptor before infection (a potential ligand for sporozoite entry) were found to lose these markers post infection, acquiring the IL-A24 phenotype (Campbell *et al*, 1994a). Expression of MHC II was very high in both *T. annulata* and *T. parva* infected cells (Spooner *et al*, 1988). *T. annulata* preferentially infected major histocompatibility complex (MHC) class II<sup>+</sup> cells (Glass *et al*, 1989). The infected cell lines of both *T. annulata* and *T. parva* expressed MHC I antigens whose BoLA specificities were the same as those of the original leucocytes used as host cells (Spooner & Brown, 1980).

Although very little work has been done to identify the potential, *in vivo*, target cells

for sporozoite invasion, the same cell subpopulations may be infected *in vivo* as well as *in vitro*. Some supporting evidence was provided by the observation that most of *in vivo* derived *T. parva* cell lines expressed T cell markers (Baldwin *et al*, 1988a). Subsequently, *T. parva* infected cells exiting in the efferent lymph of animals undergoing clinical infection were shown to co-express both CD4 and CD8 T cell markers (Emery *et al*, 1988; Spooner *et al*, 1988). The authors felt that probably CD4+ cells were initially infected and expressed CD8 on transformation.

*Theileria* parasites must undergo a recognition event for entry into target cells presumably via interactions between ligands on the parasite surface and receptors on the target cells (Tait & Sacks, 1988). Williamson *et al*, (1989) identified an antigen SPAG-1 on the surface of *T. annulata* sporozoites which was presumed to be involved in invasion as a mAb specific to SPAG-1 blocked sporozoite penetration into the target cells. SPAG-1 exhibited a remarkable degree of molecular mimicry to elastin (Hall *et al*, 1992) and it was thought that the receptor for elastin, which is expressed mainly on monocytes and macrophages, might be a ligand for sporozoite invasion. However, *T. annulata* sporozoites infected elastin receptor positive and negative cells equally well in *in vitro* experiments (Campbell *et al*, 1994a). The receptor for the entry of *T. parva* sporozoites may be related to an epitope on a 67 kiloDalton (kD) antigen on the sporozoite surface because antibodies raised against this epitope can block entry into the target cells (Musoke *et al*, 1984; Dobbelaere *et al*, 1984).

To identify the target cells for sporozoites *in vivo*, a novel pseudoafferent cannulation method was devised in the work reported here. Using this method it would be possible to collect lymph cells immediately after invasion by sporozoites.

### **2.1.6 *In vitro* cultivation**

The methods used for *in vitro* cultivation of *Theileria* parasites and their applications have been reviewed extensively by Brown (1979, 1981, 1983 & 1987). Both *T. annulata* and *T. parva* cell lines can be established from infected lymphoid tissue or peripheral blood from a sick animal. They can also be initiated by culturing sporozoites with uninfected bovine leucocytes. Such transformed parasitised cell lines can be maintained in culture medium for indefinite periods without the addition of exogenous growth factors (Hulliger *et al*, 1964). This technique has proved to be an invaluable tool for later studies on *T. annulata* and *T. parva* as it enabled infected cell lines to be generated *in vitro* using cells from naive animals but without having to infect the animals themselves. The macroschizont-infected cell has been the only stage so far amenable to continuous cultivation in the laboratory. The macroschizont-infected cell lines of various theilerial species show unlimited growth potential *in vitro* (Brown, 1987) which seems to be induced by the presence of the parasite inside the cell. Infected cells stop dividing if the parasite within the cell is killed (Pinder *et al*, 1981). Bovine fibroblast cell lines can be infected with *T. annulata* or *T. parva* if co-cultured with schizont-infected cells (Brown & Gray, 1981).

Merozoites can also be produced *in vitro* (Hulliger *et al*, 1966; Shiels *et al*, 1992) but attempts to produce piroplasms from these merozoites have failed. Cultures of red blood cells containing *T. annulata* piroplasms could be maintained for 10-27 days (Conrad *et al*, 1985). The motile kinete stage of the parasite can be maintained in culture for short periods (Bell, 1980; Bell, 1984).

### **2.1.7 Clinical symptoms**

The incubation period of *T. annulata* or *T. parva* infections following natural tick

infestation was two weeks on average (ranging between 8 to 25 days) (Sergent *et al*, 1945; Neitz, 1957). In experimental infections, clinical responses to both species depended upon the dose of initial infection with tick material (Gill *et al*, 1980; Samantaray *et al*, 1980; Dolan *et al*, 1984c; Preston *et al*, 1992a; Nichani, 1995). Schizonts in lethal infections may be detected in the lymph nodes draining the site of tick infection only 2 days after infection and piroplasms in red blood cells 4 days later (Preston *et al*, 1992a). High fever and swelling of superficial draining lymph nodes are among the initial symptoms (Dschunkowsky & Luhs, 1904; Sen & Srinivasan, 1936; Uilenberg, 1981; Nichani, 1995). Other clinical symptoms include anorexia, malaise, listlessness, dyspnoea, loss of body weight and digestive disturbances including a localised ulcerative abomasitis (Dschunkowsky & Luhs, 1904; Sen & Srinivasan, 1936; Kachani, 1992; Preston *et al*, 1992a). Terminal cases show hypothermia, recumbency, rapid breathing & pulse rate (Dschunkowsky & Luhs, 1904; Sen & Srinivasan, 1936). In *T. parva*, schizonts were detected in superficial lymph nodes draining the site of tick infection on 5th to 8th day of infection and piroplasm in red blood cells 2 to 5 days later (Irvin & Morrison, 1987). Anaemia and jaundice are common terminal features of tropical theileriosis but do not normally develop in East Coast fever, (Irvin & Morrison, 1987). The mortality rate in *T. annulata* infection is 40 to 60% (Uilenberg, 1981) and in *T. parva* is 85 to 90% (Brocklesby *et al*, 1961). Animals that survive infection may undergo a prolonged period of recovery and become carriers for piroplasms and this carrier stage may flare up again in acute tropical theileriosis at times of stress or intercurrent disease (Sergent *et al*, 1945).

A cerebral form of theileriosis known as turning sickness has been reported to occur in some animals infected with *T. parva*, usually several months or even years after

the initial disease (Mettam & Carmichael, 1936; Giles *et al*, 1978). Affected animals show ataxia and circling and may die in convulsions. These symptoms are associated with the presence of large numbers of infected cells and free schizonts in brain capillaries (Moll *et al*, 1986).

#### **2.1.8 Diagnosis**

Diagnosis of *T. annulata* and *T. parva* depends upon clinical symptoms, haematological observations, detection of parasites in lymph node biopsies & blood smears and autopsy lesions in dead animals. Indirect fluorescent antibody tests (IFAT) have been used for serum antibody assays for serological surveys of infection with *T. annulata* (Burrige & Kimber, 1972) and *T. parva* (Goddeeris *et al*, 1982). IFAT test has several disadvantages as preparations can't be stored for long periods and a degree of skill is required in interpreting results. This has prompted the development of an enzyme linked immunosorbent assay (ELISA) (Kachani *et al*, 1992b; Sunder *et al*, 1993) and DOT-ELISA (Grewal *et al*, 1991; Grewal, 1992). Recently, an immunoperoxidase technique was developed for detection of antibodies against *T. annulata* (Campbell *et al*, 1994b).

More recent attempts to improve diagnostic techniques also investigated the use of DNA probes and PCR. Oligonucleotide probes detecting small subunit ribosomal RNA sequences were used to discriminate between six species of *Theileria* including *T. annulata* (Allsopp *et al*, 1993). Ben Miled *et al* (1994) have demonstrated considerable diversity amongst various *T. annulata* stocks isolated from Tunisia using two genomic DNA probes. Polymorphism was observed between isolates collected even from the same farm. Similar approaches have been used for studying polymorphism in *T. sergenti* infection as well (Matsuba *et al*, 1992). Currently, these

probes are used in research only and are not yet suitable for routine field use mainly because they require radiolabelled probes to achieve the desired level of sensitivity. The polymerase chain reaction (PCR) technique has been developed to detect *T. annulata* in infected ticks by amplifying a DNA fragment from a small ribosomal RNA gene of *T. annulata* (de Kok *et al*, 1993; Jongejan *et al*, 1994). PCR has also been used to amplify p33/34 genes from the piroplasm DNA to distinguish between *T. sergenti*, *T. buffeli* and *T. orientalis* (Kawazu *et al*, 1992).

## **2.1.9 Pathogenesis**

### **2.1.9.1 Leucopenia**

Infection with both parasites leads to development of severe leucopenia involving both polymorphonuclear cells and lymphocytes (Sharma & Gautam, 1971; Laiblin, 1978; Morrison *et al*, 1981b; Preston *et al*, 1992a; Nichani, 1995). Lymphocyte depletion has been reported from solid lymphoid tissues and blood which was associated with marked destruction of cells in the later stages of the disease. However, mechanisms involved in the destruction of cells are not clearly known. After *T. parva* infection, Wilde (1967) reported decreased granulopoiesis in the bone marrow as well as in thymus.

### **2.1.9.2 Anaemia**

Anaemia is a common finding during acute *T. annulata* infection (Sergent *et al*, 1945; Neitz, 1957; Hooshmand-Rad, 1976). Strains of *T. annulata* which had lost the ability to produce microschorizonts and piroplasms still induced anaemia (Hooshmand-Rad, 1976). These observations suggested that factors other than piroplasm may contribute anaemia. Erythrophagocytosis has been suggested as a means of erythrocyte destruction as well as the direct effect of parasitaemia on the infected animals (Neitz,



1957; Uilenberg, 1981). PBMs collected from calves lethally infected with *T. annulata* spontaneously produced TNF $\alpha$  and IFN $\gamma$  (Preston *et al*, 1993). A TNF dependent red cell destruction and dyserythropoiesis has been shown in cattle infected with *Trypanosome vivax* and *T. congolense* (Sileghem, 1994). The same effect on erythropoiesis has been shown in nude mice after chronic exposure to TNF (Johnson *et al*, 1989). These observations suggested that these cytokines may be involved in the development of anaemia.

#### **2.1.9.3 Parasite dissemination**

Following sporozoite inoculation, the parasite appears to travel through the lymphatic system as it is first seen as trophozoites and macroschizonts in the draining lymph node. The mode and place of host cell invasion by the parasite are not fully understood nor is the specificity of the *in vivo* target cells clearly defined. Dissemination seems to be quite rapid as surgical removal of the DLN, only two days after inoculation of *T. annulata* sporozoites (Campbell, 1995), had little or no effect on the subsequent course of infection. These results suggest that the parasite may disseminate to more than one lymph node from the site of inoculation. Detection of *T. parva* schizonts in the draining lymph node before non-draining lymph nodes (Morrison *et al*, 1981b) was thought to reflect the confinement of infection in the draining node because of its proximity to the site of inoculation (Irvin & Morrison, 1987).

#### **2.1.9.4 Histological studies**

Histological examination of the draining lymph node during the initial stages of infection after inoculation of *T. annulata* sporozoites in a naive animal revealed areas of blasting cells in the medulla by day 4 and throughout the node by day 8 followed

by a total destruction of normal lymphoid architecture with the loss of existing germinal centres (Campbell, 1995). During the terminal stages of tropical theileriosis, total cell depletion from the paracortex and medulla was observed with large numbers of infected and blast cells around the follicles (Eisler, 1988). Other internal organs showed signs of congestion and haemorrhage (Sergent *et al*, 1945). In *T. parva* infection, an initial phase of blastogenesis was observed following sporozoite inoculation, which was followed by appearance of large numbers of infected cells (Barnett, 1968). Uninfected blasting cells were more prominent in the paracortical zone (DeMartini & Moulton, 1973). Blasting infected cells were more numerous in T-dependent areas than in B-dependent areas. Germinal centres were observed to undergo a rapid involution, however some infected cells were observed within germinal centres (Morrison *et al*, 1981a). There are no reports of histological studies on the DLN after immunisation with *T. annulata* cell lines.

## **2.1.10 Immune responses**

### **2.1.10.1 Immune responses to ticks**

The host response to tick antigens involves a local inflammatory reaction; the extent and complexity of which depends on the species of the tick and whether or not the host has been sensitized previously (Allen, 1973). Responses in rabbits induced by a natural tick vector for *T. annulata* include infiltration of neutrophils, mononuclear cells, eosinophils and basophils at the site of bite. The mediators released by mast cells, basophils and eosinophils are considered to be the main effectors of resistance to the tick (Gill & Walker, 1985). However, it is not known whether cellular infiltration is beneficial either to the host or the parasite. Immunising animals against tick gut antigens has given promising results against the one host tick, *Boophilus microplus* (Willadsen & Kemp, 1988). However immunity to other species of two and

three host ticks would have to be effective against different tick stages and over a shorter time period (Morrison, 1989).

#### **2.1.10.2 Immune responses to sporozoites**

The sporozoite is the first stage of the parasite and is exposed to the host immune system for only a very short time (Brown *et al*, 1978; Jura *et al*, 1983a). Sporozoite neutralising activity was found in sera from recovered animals and from animals with multiple infections and was equally effective against different isolates of *T. annulata* (Gray & Brown, 1981). Such immune serum was able to inhibit the entry of sporozoites into uninfected leucocytes *in vitro* (Gray & Brown, 1981; Preston & Brown, 1985; Ahmed *et al*, 1988). However antisera from *T. parva* infected animals did not neutralise the infectivity of *T. annulata* sporozoites, *in vitro*, implying that the active factor in immune serum was species specific (Gray & Brown, 1981). Upon further investigation of the antiparasitic activity of immune sera, two effects were observed (Preston & Brown, 1985): serum could inhibit the invasion of the target cell by the sporozoite and/or suppressed the establishment and transformation of trophozoite-infected and macroschizont-infected cells. Williamson *et al*, (1989) raised mAbs against *T. annulata* sporozoites which neutralised sporozoite infectivity *in vitro*. One of these mAbs (1A7) recognised a sporozoite surface antigen (SPAG-1) on geographically distinct stocks of the parasite. The gene coding for this protein was expressed to a significant degree only in sporoblasts and sporozoites and not in schizonts or piroplasms. Meanwhile another *T. annulata* sporozoite surface antigen (SPAG-2) recognised by a mAb 4B11 has also been described. The gene coding for this protein was cloned and expressed as a fusion protein. Hyper immune serum against this protein raised in rabbits neutralised sporozoite invasion *in vitro* and recognised an antigen on sporozoites and schizont infected cells (Knight *et al*, 1994).

SPAG-1 has been shown to bind to T cells, B cells and monocytes whereas SPAG-2 showed a high specificity for binding to monocytes (targets for *T. annulata* infection)(Katzner *et al*, 1994).

Antibodies capable of neutralising infectivity of *T. parva* sporozoites were demonstrated in cattle immunised against East Coast fever by the infection and treatment method and subsequently challenged with infective ticks several times (Musoke *et al*, 1982). Sera from immunised cattle neutralised infectivity of sporozoites of other *T. parva* stocks (Musoke *et al*, 1984). MAbs generated against sporozoites of *T. parva* also neutralised sporozoite invasion of host cells *in vitro* (Dobbelaere *et al*, 1984; Musoke *et al*, 1984). Immune sera and mAbs recognised an antigen (p67) of molecular weight of 67kD on the surface of sporozoites (Musoke & Nene, 1990; Iams *et al*, 1990). The gene coding for the p67 protein was transcribed only in the sporozoite stage. This gene is present in all stocks of *T. parva*. Partially purified recombinant antigen (NSI-p67) induced sporozoite neutralising antibodies when inoculated into susceptible animals and protected six out of nine cattle on homologous challenge. It was suggested that the elimination of parasite occurred before the schizonts were established. This recombinant antigen is a candidate for sporozoite vaccine (Musoke *et al*, 1992). In another group of 11 animals immunised with NSI-p67, 6 were protected against heterologous Marikebuni stock of *T. parva*, indicating some cross immunity (Musoke *et al*, 1993), as Marikebuni and Muguga (from where NSI-p67 was derived) stocks fall into different cross-protective groups (Morzaria *et al*, 1987). Further sequence analysis of PCR amplified p67 gene product from six different stocks of the parasite showed no variation, making it theoretically possible to cross-protect amongst various *T. parva* stocks (Musoke *et al*, 1993) with the NSI-p67. In a recent report, 23 animals were immunised with NSI-p67. Eleven

were challenged with heterologous Marikebuni stock and 12 with homologous Muguga stock of sporozoite along with 20 control animals (Nene *et al*, 1996). Six of the 11 and 7 of the 12 cattle were clinically immune whereas all controls underwent severe disease. Use of better adjuvants might improve the immunising efficacy of these recombinant proteins. In the immunisation experiments with NSI-p67, an immune response against sporozoites alone might not be adequate to prevent infection of host cells as a few sporozoites might escape neutralisation and develop as schizonts. The status of recombinant sporozoite vaccine was briefly reviewed by Musoke & Nene (1990).

#### **2.1.10.3 Immune response to macroschizont-infected cell lines**

The schizont stage of the parasite is pathogenic in tropical theileriosis as well as in East Coast fever. Two types of responses are explained below.

##### **2.1.10.3.1 Antibody responses**

Animals undergoing immunisation against *T. annulata* or recovering from the infection develop circulating antibodies to all stages of the parasite (Kachani *et al*, 1992a, b). High IFA titres were detected in cattle after exposure to the parasite (Pipano & Cahana, 1969) and after vaccination with attenuated cell lines (Shukla & Sharma, 1991). Using sera from recovered and cell line immunised animals, Kachani *et al*, (1992a) demonstrated three antigens between 71 and 73 kD by Western blot analysis which were common to sporozoite, schizont and piroplasm stages of *T. annulata*. Another 32 kD antigen was found to be specific to piroplasm stage only. Several observations suggested that humoral responses may not have any protective role particularly against schizont stage. Immune sera recognised the intracellular parasite as shown on IFA test slides, but did not detect parasite associated antigens

on the host cell surfaces (Creemers, 1983; Shiels *et al*, 1989; Glascodine *et al*, 1990). Passive transfer of immune serum from immunised cattle (recovered after infection) to susceptible animals did not prevent development of either *T. annulata* (Sergent *et al*, 1945; Dhar & Gautam, 1978; Samad *et al*, 1984) or *T. parva* infection (Muhammed *et al*, 1975), although serum from immune cattle exhibited antibodies against all stages of the parasite (BurrIDGE & Kimber, 1972). Experiments using lysates or inactivated schizont material to immunise animals have not induced a protective response in either *T. annulata* (Pipano *et al*, 1977) or *T. parva* (Wilde, 1967; Emery *et al*, 1981b) infection. Similarly, attempts to immunise against *T. parva* using extracts of piroplasm were unsuccessful (Wagner *et al*, 1974). Serum from cattle, recovered from *T. annulata* infection, was not opsonic for parasite infected cells and did not lyse these cells in the presence of complement (Ahmed *et al*, 1988). Similarly, Duffus *et al*, (1978) demonstrated that serum from *T. parva* immune cattle did not lyse infected cells. However, the serum from immune cattle showed macrophage mediated antibody cell cytotoxicity (Preston & Brown, 1988). Generation of antibodies against schizonts and piroplasm is a useful diagnostic aid for conducting serological surveys and assessing response of animals after immunisation.

#### **2.1.10.3.2 Cell mediated responses**

Studies on *T. annulata* showed that calves recovering from infection produced cytotoxic cells which recognised parasite antigen in conjunction with self MHC class I molecules (Preston *et al*, 1983). Two peaks of cytotoxicity were observed during recovery from *T. annulata* infection. The first peak was BoLA restricted and probably analogous to cytotoxic T lymphocytes, while the second peak was not MHC restricted and probably analogous to natural killer cells. Innes *et al*, (1989a) showed cell mediated cytotoxic activity against *T. annulata* infected cells after primary

immunisation with autologous or allogeneic *T. annulata* infected cell lines. Animals immunised with allogeneic cell line initially developed (around day 9) a strong cytotoxic response directed against the MHC antigens of the immunising cell line followed by MHC class I restricted cytotoxic (around day 23) against autologous infected cells. In contrast, animals immunised with autologous infected cells developed severe clinical reactions and only a low level of cytotoxic activity against parasite infected autologous cells, which was not MHC restricted up to day 20. Two phases of immune responses were observed in the efferent lymph after immunisation of cattle with an allogeneic cell line (Nichani, 1995). Both these phases of responses appeared earlier in the efferent lymph than in the PBM. This study indicated that immune responses developed in the DLN. Sera specific for bovine MHC class I antigens blocked the cytotoxic activity further confirming it to be MHC class I controlled genetically restricted response (Innes, 1988).

Cattle undergoing lethal *T. parva* infection exhibited cytotoxic cells which killed allogeneic infected cells and xenogenic murine cells but not autologous infected cells, in PBMs, whereas animals immunised by infection and treatment generated genetically restricted cytotoxic cells which killed autologous infected cells (Emery *et al*, 1981a). Fractionation of effector PBMs into different cell populations revealed that the cytotoxic activity was mediated by T cells (Emery *et al*, 1981b). These studies revealed that generation of genetically restricted cytotoxic cells against autologous *T. parva* infected cells was a feature of immune animals or of animals undergoing immunisation and directly correlated to protection from lethal sporozoite challenge. This mechanism was not stimulated in animals undergoing acute *T. parva* infection which seemed to generate a non-specific cytotoxic T cell responses.

Additional evidence of genetically restricted cytotoxic cells came from the experiments of Morrison *et al*, (1986) who showed that cytotoxic cells, in ECF immune cattle following sporozoite challenge, killed only autologous macroschizont-infected cells or infected cells from other animals expressing matched or half matched BoLA types. The response was found to be biased towards one or other BoLA phenotype. A mAb specific for bovine CD8 cells partially blocked cytotoxic activity. Cytotoxic activity was also blocked by an anti MHC class I specific mAb but not by anti MHC class II mAb indicating that the response was produced by MHC class I restricted cytotoxic T lymphocytes (CTL) of CD8 lineage (Morrison *et al*, 1987). The cytotoxic response showed some parasite strain specificity as well.

#### **2.1.10.4 Immune responses to merozoites and piroplasm**

Antibody responses to piroplasm have been observed in cattle recovering from infection or undergoing immunisation with either *T. annulata* (Pipano, 1974) or *T. parva* (BurrIDGE & Kimber, 1973). Immune serum from *T. annulata* infected animals has been shown to react with free merozoites produced by lysis of erythrocytes (Ahmed *et al*, 1988). Immune responses against erythrocytic stages are not considered very important in relation to controlling the parasite, since these occur very late in the course of the disease. Generation of immunity against this stage might be important when developing sub-unit vaccines comprising antigenic determinants from different parasite stages or blocking transmission of the parasite to the tick vector.

#### **2.1.11 Current control measures: vaccination against *Theileria* spp.**

The control of theileriosis by vaccination has been reviewed extensively (Brown, 1981; Morrison *et al*, 1986; Tait & Hall, 1990; Dolan & McKeever, 1992; Norval *et al*, 1992; Pipano, 1995). The first attempts included suspensions of lymph nodes and



spleen homogenates collected from *T. parva* infected animals to immunise susceptible cattle (Theiler, 1911; Spreull, 1914). This method was soon discontinued because many animals developed clinical theileriosis following immunisation and because of the risk of transmission of other diseases. The same approach was used for *T. annulata* (Sergent *et al*, 1924), however it was also discontinued because of the risk of transmission of other diseases, failure to provide constant infective material and the death of some susceptible animals. With advances in chemotherapy and *in vitro* cultivation of theilerial parasites, attention focused on the development of prophylactic methods using schizont derived cell culture vaccines (Pipano & Tsur, 1966) and simultaneous infection with sporozoites and treatment with a suitable drug (Neitz, 1953; Cunningham *et al*, 1973).

#### **2.1.11.1 *Macroschizont-infected cell line vaccination***

This method of vaccination is the only method of immunoprophylaxis against *T. annulata*. Early studies showed that with loss of virulence in culture, the parasite lost the capacity to produce piroplasms but retained its immunological properties (Pipano & Israel, 1971; Pipano *et al*, 1973) and thus did not provide a source of infection for ticks (Samish *et al*, 1984). Inoculation of such attenuated schizonts culture resulted in milder clinical symptoms and lower parasitaemia in susceptible animals (Pipano & Tsur, 1966; Hashemi-Fesharki & Shad-del, 1973; Gill *et al*, 1976b; Singh *et al*, 1993a; Nichani, 1995). Although immunised animals developed mild parasitological reactions on challenge with ticks or stabilates (Gill *et al*, 1976b; Pipano, 1981; Shukla & Sharma, 1991).

Attenuated cell lines used as vaccines in different countries have been developed by continuous passage in culture and tested for immunogenicity by inoculation in

susceptible calves at different passage levels. Sporozoite challenge of animals immunised with such schizont infected cell culture vaccines usually resulted in mild reactions and protection even against heterologous stocks of the parasite (Pipano, 1981; Brown, 1990). Occasionally, highly susceptible adult Friesian cows were inadequately protected and reinforcement of a second immunisation with infected tick material was recommended in such animals (Pipano, 1981).

Complete attenuation has been reported after 600-900 days in culture (Pipano and Tsur, 1966; Singh, 1990), after 60 (Hashemi-Fesharki, 1988), after 250 (Ozkoc and Pipano, 1981) passages in culture. The development of cell culture vaccines against tropical theileriosis has been reviewed by Pipano (1977; 1981; 1984). Pipano (1974) reported the protection of young, adult beef cattle and female animals up to the age of first pregnancy after inoculation with  $1-2 \times 10^6$  cells. This vaccine could be either used fresh ( $3-5 \times 10^6$  cells) or after cryopreservation ( $10^7$  cells) and had a shelf life of four days at  $4^\circ\text{C}$ . (Pipano, 1981). Cell culture propagated schizont vaccines have been used efficiently in many countries where *T. annulata* is endemic including Iraq (Hooshmand-Rad, 1973), Iran (Hashemi-Fesharki & Shad-Del, 1973; Hashemi-Fesharki, 1988), China (Gansu Provincial Institute of Veterinary Medicine, 1975; Wenshun *et al*, 1982), Russia (Stepanova *et al*, 1977), Israel (Pipano, 1978; 1981), Turkey (Ozkoc & Pipano, 1981), India (Singh, 1990; Shukla & Sharma, 1991), and Morocco (Ouhelli, 1991).

Cattle immunised with a cell culture vaccine were protected on challenge with sporozoite stocks from remote geographical areas in some experiments (Preston & Brown, 1988; Innes *et al*, 1989c; Nichani, 1995) but poorly protected in others (Ozkoc & Pipano, 1981; Subramanian *et al*, 1987).

The duration of immunity in absence of reinfection after cell line immunisation has not been fully investigated. There are some reports indicate the immunity conferred by schizonts wanes with time in the absence of re-challenge (Sergent *et al*, 1945; Pipano, 1977; Ouhelli *et al*, 1994). Pipano (1977) reported that cattle immunised with attenuated schizonts were protected when challenged after 18 months. Animals vaccinated with cell culture vaccine were protected for more than one year (Singh, 1990). In a more promising report, Zablotzky (1991) found that animals were immune to challenge 3.5 years after immunisation.

Little is known about the mechanism by which prolonged culture of cell lines lead to attenuation of the parasite (Tait & Hall, 1990). It might simply be a clonal selection over period of time, in which case selection pressures will be a priority area of study (Brown, 1990). Studies on glucose phosphate isomerase isoenzyme patterns on *T. annulata* infected cell lines at very early passages revealed multiple banding pattern, but late passages and cloned cell lines only revealed a single triplet (Melrose *et al*, 1980). Reduction in virulence by prolonged cultivation of *T. annulata* infected cell lines accompanied the loss of an antigen recognised by mAb EU106 and changes in the RFLP patterns leading to selection of a particular parasite subpopulation (Sutherland *et al*, 1996). Activity of host cell proteases has been shown to be reduced upon long term culture (Baylis *et al*, 1992). However there is as yet no conclusive evidence of any relationships between the disappearance of particular antigens, alterations in gene expression in infected cells, changes in GPI pattern or protease production by infected cells.

Cell lines infected with *T. annulata* and *T. parva* express bovine leucocyte antigens (BoLA) on their surface similar to the animal from which the lines are isolated

(Spooner & Brown, 1980). During cell line immunisation, the parasite is introduced in the recipient animal within a foreign cell in the form of a graft. Therefore, histoincompatibility between cell line and the recipient may influence successful immunisation on inoculation of parasite infected cell line. An important feature for development of immunity after immunisation with allogeneic infected cell line is that infection has to transfer and establish into the cells of recipient animal (Wilde 1967; Pipano *et al*, 1977; Brown *et al*, 1978a; Emery *et al*, 1982; Innes *et al*, 1989c; Nichani, 1995), however, neither the mechanism involved nor the site of parasite transfer are yet known.

Immunisation against *T. parva* with attenuated schizonts has not been very successful as compared to *T. annulata*. Initial studies indicated that at least  $10^8$  *T. parva* infected allogeneic cells were required to induce reliable immunity (Brown *et al*, 1978a), whereas cell doses as low as  $10^2$  (Ouhelli *et al*, 1989) and  $10^1$  allogeneic cells (Brown, 1990) would infect and immunise animals against *T. annulata*. While  $10^8$  or more allogeneic *T. parva* infected cells were needed for parasite transfer and induction of immunity (Emery *et al*, 1982), as few as  $10^2$  autologous infected cells resulted in sub-patent infection and development of immunity to challenge with sporozoites (Morrison *et al*, 1981a; Buscher *et al*, 1984). Cattle inoculated with  $10^3$  or  $10^5$  *T. parva* infected MHC class I matched cells produced immunity, but animals inoculated with similar doses of MHC class I mismatched cells were not protected on subsequent challenge with sporozoites (Teale, 1983; Dolan *et al*, 1984b).

The MHC class I histocompatibility barrier does not appear to jeopardise infection and immunisation using *T. annulata* infected cell lines (Innes *et al*, 1989a). Using karyotypic analysis, it was observed that *T. annulata* infected cells would transfer and

infect cells of the recipient animal within hours of inoculation (Wilde, 1967), whereas, schizont transfer was very inefficient with *T. parva* infected cells and could take many days (Brown, 1981). Innes *et al*, (1989c) demonstrated the phenomenon of parasite transfer by immunising animals with BoLA mismatched *T. annulata* infected cells and reisolating parasite infected cells from the recipient animals which expressed recipient's BoLA type. Similar findings were observed in the efferent lymph after allogeneic cell line immunisation (Nichani, 1995). The relative ease of immunisation using allogeneic *T. annulata* infected cell lines compared to allogeneic *T. parva* infected cell is unlikely to be solely due to the effect of MHC incompatibility between cell line and recipient, as this is a common element in both cases, but more likely to involve the ability of each parasite to transfer and infect different cell populations of the recipient (Innes *et al*, 1992).

Animals immunised with killed *T. annulata* schizont material developed high antibody titres after inoculation but were not immune to sporozoite challenge (Pipano *et al*, 1977). In a more recent study, two calves inoculated with purified plasma membrane from *T. annulata* infected cell line were protected on sporozoite challenge with the same parasite stock, but the other two calves immunised with the same material were fully susceptible to challenge with a slightly different stock of the sporozoites (Chaudhri & Subramanian, 1991). In a similar study, Emery *et al*, (1986) showed that 3 out of 4 calves inoculated with purified plasma membrane from autologous *T. parva* infected cell line were protected on sporozoite challenge, whereas calves inoculated with purified plasma membrane from an allogeneic cell line were not protected. However, it is essential to ensure that no live parasite remains. In attempts to replicate these results (Brown *et al* unpublished) the only animals protected developed piroplasms, indicating the membranes had been inadequately prepared and

contaminated with infective material. The mechanism of development of immunity to purified plasma membranes from *T. annulata* and *T. parva* infected cell line is not understood and further work in this field is lacking.

#### **2.1.11.1.1 *Revaccination with macroschizont-infected cell line***

Immunity wanes after cell line immunisation leading to the necessity of revaccination and work from Israel suggested that revaccination with same cell lines may not be effective (Pipano, 1978). In Morocco (Ouhelli, 1994), animals immunised with  $10^4$  cells and reimmunised with  $10^6$  of the same cell line showed variable reactions upon challenge indicating that there are chances of failure after revaccination with the same cell line. In Turkey, reimmunisation with the same cell line is being used but there is no information of its efficacy (M. Gunay quoted by Nichani, 1995).

Recently, Nichani (1995) skin grafted the animals several times to produce an anti-MHC response before revaccination with the cell line isolated from the animal used as the skin donor. He then showed that the preexisting anti-MHC immune response blocked the parasite specific immune response after reimmunisation with the same cell line. This observation suggested that the reimmunisation with the same cell line may not be useful in the field conditions.

#### **2.1.11.2 *Vaccination by sporozoite infection and chemotherapy***

A relatively costly and not very efficient method of vaccination, commonly known as "The Infection and Treatment" method, has been developed mainly for *T. parva*. Various reviews of this method can be found in (Cunningham, 1977; Radley, 1981; Brown, 1985, Pipano, 1995). The discovery by Neitz (1953) that *T. parva* can be mitigated by repeated treatment with chlortetracycline led to the development of a

method which was later on known as "the infection and treatment" method of vaccination. It was observed that treatment resulted in a decrease and disappearance of schizonts followed by recovery and development of solid immunity to reinfection. Similar results were reported while treating the animals with oxytetracycline after infection (Neitz, 1957). Further development of this technique was possible after methods for producing cryopreserved sporozoite stabilates were developed (Cunningham *et al*, 1973). Cryopreservation of stabilates allowed infection of cattle with a particular pre-determined dose along with administration of long acting oxytetracycline (Radley *et al*, 1975a; Radley, 1981).

A similar technique has been developed for immunisation against *T. annulata* as well, but only under experimental conditions. The technique has been used to immunise cattle successfully against tropical theileriosis (Gill *et al*, 1976; Jagdish *et al*, 1979; Pipano, 1981; Khanna *et al*, 1983). All these experiments used oxytetracycline although later on this proved not to be a drug of choice for immunising very young calves (Mallick *et al*, 1987). With the development of parvaquone (Clexon, Wellcome) and buparvaquone (Butalex, Mallincrodt Animal Health Ltd.) (McHardy *et al*, 1985a, b), cattle have been immunised successfully against *T. parva* by the inoculation of infective stabilates and treatment with parvaquone (Dolan *et al*, 1984a) or buparvaquone (Mutugi *et al*, 1988; Young *et al*, 1990). The chemoimmunoprophylactic efficacy of buparvaquone against *T. annulata* infection was demonstrated and found to be better than long acting oxytetracycline (Dhar *et al*, 1990). Very young calves (below four weeks of age) were successfully immunised using infective tick material equivalent to as low as 2 infected acini (Dhar *et al*, 1990) and to as high as 30 ticks (Kumar *et al*, 1990a) along with buparvaquone treatment. Calves inoculated only with buparvaquone and kept in heavily tick infested

paddocks also became immune indicating that the drug alone will protect calves born in the disease season for at least a month (Kumar *et al*, 1990b). Young calves immunised in this way were immune to subsequent potentially lethal challenge immediately after immunisation (Rana & Dhar, 1993) and even if they were already undergoing the incubation period of the disease (Dhar & Rana, 1993). These reports suggest that immunisation of very young calves by this method can be undertaken even after onset of the disease season. Studies on *T. parva* showed that parvaquone given at early stages of infection inhibited the development of immunity (Dolan *et al*, 1988). The dose of *T. parva* sporozoites has to be chosen carefully while using buparvaquone for simultaneous treatment, since too high a dose can break the protection and too a low dose may not produce immunity (Ngumi *et al*, 1992). It may be concluded that a wider sporozoite dose range can be used with buparvaquone than with oxytetracycline.

This "infection and treatment" method of immunisation has some drawbacks: production of sporozoite is very labour-intensive and expensive, different batches of ticks vary in infectivity, vials of the same cryopreserved stock can also produce variable reactions in inoculated animals. Moreover, Butalex, the only commercially available preparation of Buparvaquone, is very costly for a farmer in a developing country. Another problem is immunological heterogeneity as found amongst various *T. parva* stocks. Immunisation against one stock of *T. parva* did not protect against all stocks of the parasite (Young *et al*, 1973; Radley *et al*, 1975b). Some *T. parva* stocks require higher levels of drug in order to control infection than others (Radley, 1981).



## **2.2 BOVINE LYMPHOID SYSTEM AND RESPONSES TO INFECTION**

Lymphocytes migrate from the blood to the lymphoid and nonlymphoid tissues by several routes (Fig. 2.3). More than 90% of T cells in the efferent lymph are naive cells whereas more than 90% of T cells in the afferent lymph are memory cells (Mackay *et al*, 1990). Naive T cells leave the blood through the high endothelial venules (HEV) and enter lymphoid tissue; they return to the blood via the efferent lymphatics.

Immune responses to antigens in the lymph node can be studied by lymphatic cannulation techniques (Hall & Morris, 1962). Immune responses in afferent or efferent lymph to various exogenous protein antigens (Hall & Smith, 1971; McConnell & Hopkins, 1981) and microorganisms (Hall *et al*, 1967; Emery, 1981b; Entrican *et al*, 1991a; Bird *et al*, 1993; McKeever *et al*, 1994) have been extensively studied in sheep and, to a lesser extent, in cattle. A recent study reported in detail the immune responses to *T. annulata* which occurred in the efferent lymph of cattle after infection or cell line immunisation (Nichani, 1995).

### **2.2.1 Draining of antigen from the site of inoculation to the lymph node**

Foreign antigens or microorganisms rapidly enter the lymph stream and travel via afferent lymphatics to the regional lymph node (Morris, 1972). Studies on the migration of cells from the site of antigen deposition to the lymph node are facilitated by the technique of pseudoafferent cannulation where afferent lymphatics was allowed to anastomose with the efferent lymphatics after surgical removal of the lymph node. Cannulation of efferent lymphatics at this stage permits collection of afferent lymph (Emery *et al*, 1987). Bovine afferent lymph contains around 50% T lymphocytes, 20-25% B lymphocytes and 23% non-lymphoid cells (Emery *et al*, 1987), but their role

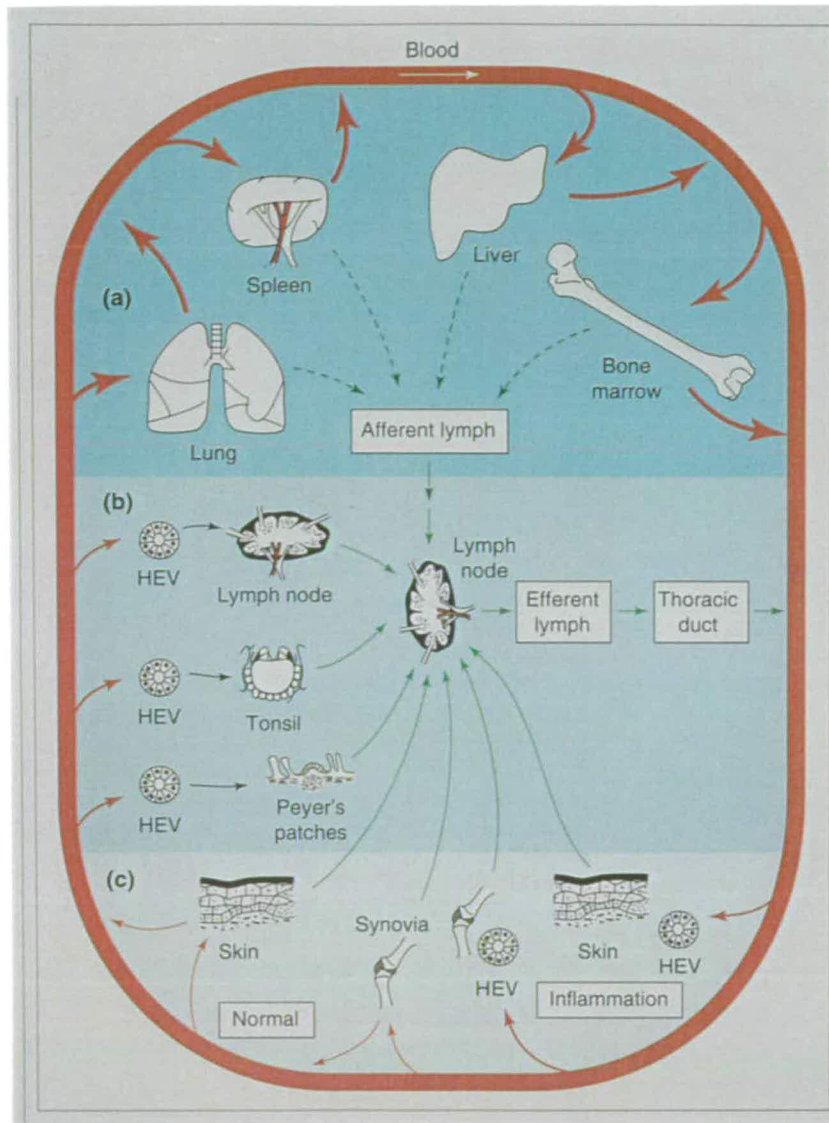


Fig. 2.3 Lymphocyte migration routes between blood and lymphoid/nonlymphoid tissues. (Figure adapted from *Immunology Today*, 17(6), 280).

in the immune response is unclear. Part of this thesis deals with the immune responses in the afferent lymph.

Increase in the output of lymphocytes was observed in the afferent lymph draining granulomatous and DTH lesions (Hay *et al*, 1973) whereas no changes have been reported after infection with *Trypanosoma spp* (Flynn *et al*, 1994). Following primary inoculation of soluble protein antigens, afferent lymph dendritic cells transiently expressed increased levels of CD1 (Hopkins *et al*, 1989). Secondary antigen challenge in already primed sheep led to an initial drop in cell output in afferent lymph for 1-3 days, followed by approximately five fold increase by day five. This was associated with increased output of MHC class II<sup>+</sup> T cells in afferent lymph probably because of increased recruitment of activated or memory T cells at the site of antigen challenge. A quantitative increase in expression of MHC class II on dendritic cells and lymphocytes and CD1 expression on dendritic cells was also observed. These changes in dendritic cells were associated with an increased antigen presenting ability to activated T cells *in vitro* (Hopkins *et al*, 1989).

No report is available regarding the functional capacity of afferent lymph in bovine tropical theileriosis. In bovine trypanosomiasis, no changes in CD2 and a constant ratio of CD4 & CD8 with a marked decrease in  $\gamma\delta$ T cells have been reported (Flynn *et al*, 1994). Suboptimal lymphocyte activation due to immune suppression was proposed to be the cause of immune failure in bovine trypanosomiasis.

### **2.2.2 Immune responses in the draining lymph node**

Part of the work in this thesis deals with immune responses in the DLN. Lymph nodes are specialised and well compartmentalised structures which act as filters for

foreign antigen material entering the lymphatic system and provide a favourable environment for the interaction of antigen presenting cells (APC) and T cells which are essential for the generation of immune responses (Breel *et al*, 1988). Bovine lymph nodes are organised into distinct areas: the follicles (B cell area) and the paracortex (T cell area). Multiple afferent lymphatics enter the lymph node capsule and empty into the subcapsular sinus (Fig. 2.4) (Morrison *et al*, 1986a). Naive T cells enter the node from peripheral blood through specialised post-capillary venules also called HEV in paracortex (Bogen *et al*, 1991). HEVs and the paracortex are the sites for T cell activation (Bogen *et al*, 1991 & 1993). T cells have been shown to be essential for the formation of B cell germinal centres (GC) (Jacobson *et al*, 1974) and T cells are supposed to be antigen specific in the GCs (Kathleen *et al*, 1993). GC form in the B cell follicles after antigen challenge and are the site where B cells undergo proliferation and antibody isotype switching is generated (MacLennan, 1994a). B cells require T cell help, first in initial stimulation to proliferate which is mediated through interaction between CD40 on the B cell and its ligand on T cells (Noelle *et al*, 1992; Armitage *et al*, 1993; Lederman *et al*, 1992) and later in the subsequent control of proliferation and antibody class switching through cytokine production (Armitage *et al*, 1993; Donckier *et al*, 1994). Following antigen challenge, activated T cells migrate toward towards B cell follicles (Bogen *et al*, 1991 & 1993). This is followed by B cell responses. Activated T cells make either predominantly IFN $\gamma$  or IL-4 (Ronchese *et al*, 1994). IL-4 has been shown to induce humoral responses (Abbas *et al*, 1991). Cytokine production at the site of inoculation has been reported to influence T cell responses in nodes as IFN $\gamma$  producing NK cells has been reported at the site of challenge (Bogen, *et al*, 1993).

Recently Campbell (1995) suggested a possible role for IFN $\gamma$  in the destruction of

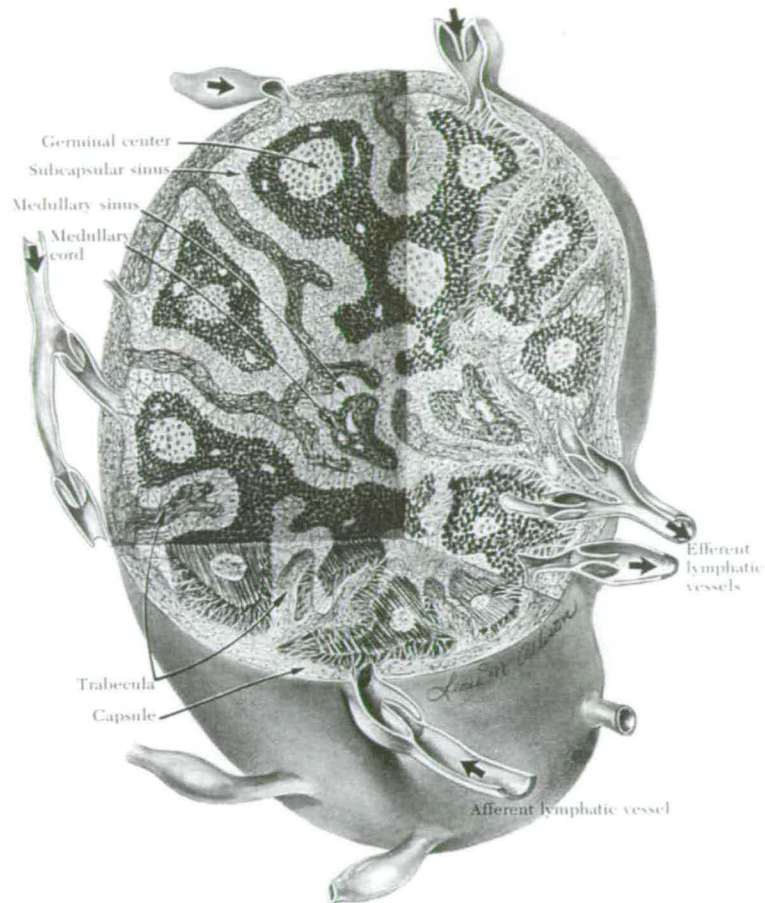


Fig. 2.4 Various compartments of a lymph node as seen histologically. Arrows indicate the direction of lymph flow. (Figure adapted from "Text book of histology." Editors: Leeson, CR; leeson, TS and Paparo, AA. W.B. Saunders Co.,1985).

GC in the DLN after *T. annulata* infection. He observed proliferating B cells in the dark zone which never differentiated to enter the light zone. At the same time a loss of IL-4 which is essential for B cell differentiation was observed. Campbell (1995) therefore suggested that IFN $\gamma$  was responsible for the loss of IL-4 leading to destruction of GC.

The above processes have not been investigated in animals undergoing immunisation with *T. annulata* cell line. However, IFN $\gamma$  & IL-4 would be expected to occur as high antibody titers have been reported after immunisation (Shukla & Sharma, 1989, 1991; Kachani, 1992a, b) and IL-4 has been shown to induce humoral responses (Abbas *et al*, 1991). In this thesis an attempt has been made to elucidate the role of IFN $\gamma$  and IL-4 in cattle after immunisation with *T. annulata* cell lines.

Immune responses to a particular antigen are disseminated in the body through efferent lymphatics which enter the circulation through the thoracic duct (Fahy *et al*, 1980). If the antigen is inoculated into only one lymph node and all the cells exiting in the efferent lymph are drained from the challenged node, then the establishment of systemic immunological memory to that particular antigen is prevented and the animal remains naive as far as that antigen is concerned (Hall *et al*, 1967).

### **2.2.3 Dissemination of immune responses through efferent lymph**

Lymphocytes are the predominant cellular constituents of efferent lymph which in sheep contains about 20-30% B cells, 70-80% T cells and no macrophages or monocytes, and exit in large numbers from the node (Cahill *et al*, 1978). "Resting" efferent lymph in normal cattle contains 58.5% CD4 cells, 21.5% CD8 cells, 17.5% IgM<sup>+</sup> B cells (Emery *et al*, 1988, Nichani, 1995) and 2-3% IL-A24<sup>+</sup> monocyte/

macrophages (Nichani, 1995)

#### **2.2.3.1 Cell concentration and flow of efferent lymph**

A single resting lymph node of approximately 1gm. has an average output of 5 ml lymph and 30-50 million lymphocytes per hour in sheep (Trinka & Cahill, 1980). There is a marked increase in the blood supply to the node after antigen stimulation leading to an increased entry of fluid and lymphocytes to the node (Hay & Hobbs, 1977). An increased supply of cells but a reduced exit of cells from the node is a distinct phenomenon occurring independently and mediated by different mechanisms (Cahill *et al*, 1976). Emery (1981b) observed an increased rate of flow, by 2 to 3 fold, around 8 to 12 days after *T. parva* infection in cattle. Whereas a massive 8 to 10 fold increase was observed after *T. annulata* infection but only a 2 fold increase after *T. annulata* cell line immunisation (Nichani, 1995). Nichani (1995) also reported a massive increase in cell output after infection but only a slight increase after immunisation. A 2-3 fold increase in lymph and up to a 10-fold increase in lymphocyte output was observed in the efferent lymph of cattle draining site of *Trypanosome congolense* infection (Akol & Murray, 1986). Whether the increased blood flow is mediated by the release of cytokines or vasoactive substances is not clearly known. PGE<sub>2</sub> may be involved in the increased blood flow to the node and enhance vascular permeability to fluid and lymphocytes (Hopkins *et al*, 1981). Other potential mediators like prostaglandins (Trinka & Cahill, 1980), macrophage migration inhibitory factors, mitogenic factors (Hay *et al*, 1973) and bovine cytokines might be responsible for increased output of fluid in the efferent lymph.

Resting efferent lymph contains only a few blast cells which increased in the lymph node in response to antigen and appeared in efferent lymph between 4-5 days (Trinka

& Cahill, 1980). Blasting cells in the efferent lymph have been reported in response to infections in *T. parva* (Emery, 1981b), *T. annulata* (Nichani, 1995) and after *Toxoplasma gondii* infection in sheep (McColgan *et al*, 1987). Similarly blasting cells were observed after *T. annulata* cell line immunisation in cattle (Nichani, 1995) but the intensity of this response was not so severe as observed after infection with sporozoites of *T. annulata*.

#### **2.2.3.2 Lymphocyte recirculation**

Once a primary immune response is initiated in a DLN, activated lymphocytes leave the node via efferent lymph. They reach the general circulation through the thoracic duct and are disseminated throughout the body. Memory cells migrate selectively through peripheral tissues, such as skin, where there are more chances to encounter the antigen, and are drained back to the lymph node via afferent lymph (Mackay *et al*, 1989). Some studies suggest that a proportion of cells migrate in a tissue specific manner, for example Washington *et al*, (1988) extracted small CD4<sup>+</sup> cells in a normal lymph node more efficiently than other subsets. Small CD4<sup>+</sup> (possibly naive cells) also showed some specificity to recirculate through the same tissue (Abernethy *et al*, 1991).

How naive and memory lymphocytes take different recirculation routes is not properly understood. Such differences presumably depend upon differential homing receptor molecules (Mackay *et al*, 1989). A three step model of migration has been suggested (Mackay, 1993). The first step involves primary interaction between L-selectin on the surface of cells with HEV, followed by a second step involving some activation signal, probably by a cytokine, leading to changes in  $\beta$  integrins leading to a strong adhesion and flattening of the cell. The final step involves transendothelial



migration. Antigen challenged lymph node showed an increase in the migration of memory type  $\beta 1$  integrin<sup>+</sup> T cells, and induction of an inflammatory adhesion molecule VCAM-1 on the endothelium (Mackay *et al*, 1992a). Increase in VCAM-1 on HEV might be one of the molecular mechanisms that accounts for the transient increase of memory type T cell traffic through the node.

Recent studies have cast some doubts as to whether naive and memory T cells migrate to specific organs. By following migration of labelled sheep lymphocytes *in vivo*, it has been shown that naive and memory T cells migrate in substantial numbers into lymphoid and nonlymphoid organs (Washington *et al*, 1995). The latest information about the circulation of naive and memory cell have recently been reviewed by Westermann & Pabst (1996).

## **2.3 BOVINE LEUCOCYTE MARKERS**

Various bovine leucocyte differentiation antigens have been tested and compared in two international workshops (Howard *et al*, 1991a; Howard & Naessens, 1993). MAbs against bovine antigens that were homologous to a human CD antigen were assigned the same number with a prefix "Bo" to facilitate comparison of immunological observations between species. In the case of clusters for which there was no obvious equivalent human CD antigen, a new WC (workshop cluster) number was designated (Naessens, 1993). The leucocyte antigens which are relevant to this thesis are discussed below.

### **2.3.1 CD2**

This antigen is expressed on T cells and is a receptor for spontaneous rosette formation with sheep red blood cells (RBCs). Activation of T cells through CD2

molecule has been shown to occur through binding to the functional ligand known as CD58 (Davies *et al*, 1987). Analysis of CD2 in humans have shown a 45-50 kD transmembrane glycoprotein which is one of several accessory molecules involved in adhesion and activation of T cells and transduction of activation signals across cell membrane (Bierer *et al*, 1989). The mAbs to bovine CD2 precipitate a 50-60 kD molecule and inhibit rosette formation with sheep RBCs (Davies *et al*, 1988). Bovine CD2 is expressed on the majority of CD4 and CD8 cells in PBM and a very small proportion of CD4<sup>+</sup>/CD8<sup>-</sup> cells, but not on WC1<sup>+</sup> T cells, B cells or monocytes/macrophages. In immunohistological studies, MAbs to the bovine CD2 molecule stain the majority of thymocytes in both the cortex and medulla of the thymus except some lymphocytes immediately beneath the thymic capsule and T dependent areas in the lymph node paracortex and a few cells in B dependent follicular areas (Davies *et al*, 1988).

### **2.3.2 CD4 and MHC class II**

The CD4 molecule in cattle was first identified in 1986 (Baldwin *et al*, 1986). The mAbs recognising bovine CD4 precipitate two polypeptides of approximately 52 and 55 kD. CD4 is expressed on about 70% bovine thymocyte and 30% PBMs but not by monocytes or B cells. The expression is restricted to CD2<sup>+</sup> and  $\alpha\beta$  TCR<sup>+</sup> cells which are found in large number in T dependent areas but only a few cells are seen in follicular areas (Baldwin *et al*, 1986; Bensaid & Hadam, 1991). CD4 cells do not express WC1, a marker found on  $\gamma\delta$ T cells (Clevers *et al*, 1990).

Bovine CD4 is found on cells of helper phenotype (Baldwin *et al*, 1986) which have the ability to produce self growth factor (cytokines) in the absence of other cell types, while not being able to mediate cytotoxicity. While bovine CD4 cells can act as CTL

(Baldwin *et al*, 1992) this is not thought to be their primary function. Long term cultured bovine CD4 and CD8 T cell line express receptors for interleukin-2 (IL-2) and IL-2 alone can induce DNA synthesis in these cells (Takamatsu *et al*, 1990).

CD4 T cells recognise antigens presented by APC in the context of MHC class II molecule. APC have been shown to be essential for isolating antigen specific bovine CD4 cells *in vitro* (Glass & Spooner, 1989). The responses of T cells to antigens presented through MHC class II molecules has been useful in identifying class II function. The responses of MHC class II to foot and mouth disease virus peptide, have been shown variable in cattle but not in inbred guinea pigs (Bittle *et al*, 1982; DiMarchi *et al*, 1986). Glass (1991) have shown that some FMDV peptides are presented less efficiently than others. The above studies led to the design of peptides which are efficiently presented by a wide range of MHC class II types (Van Lierop *et al*, 1995).

Two subsets of CD4 T cells have been defined in mouse which have been designated as T helper (T<sub>H</sub>) 1 and 2 (Mosmann *et al*, 1986). T<sub>H1</sub> subset produces IL-2 and IFN $\gamma$  cytokines which help cell mediated immune responses whereas T<sub>H2</sub> subset produces IL-4 and IL-10 which help humoral immune responses (Mosmann *et al*, 1986; Scott *et al*, 1988, Coffman *et al*, 1991). The role of these cytokines has been demonstrated in various parasitic infections.

In cattle, PBM secretes both type of cytokines when stimulated with *T. annulata* infected cells. Cytokines were produced in both CD4<sup>+</sup> and CD4<sup>-</sup> populations, but production in CD4<sup>-</sup> populations (T<sub>H1</sub>) was dependent upon the presence of CD4<sup>+</sup> cells (Campbell, 1995).

### 2.3.3 CD8 and MHC class I

CD8 antigen is expressed on a subpopulation of T lymphocytes which show a MHC class I restricted cytotoxic effector function (Maddox *et al*, 1985). CD8 consists of two polypeptide chains  $\alpha$  and  $\beta$  with a molecular weight (mw) of 34-35 kD respectively. The antigen binds to a non-variable portion of the  $\alpha_3$  domain of MHC class I molecule on APC and stabilises the interaction between the two cells (MacHugh & Sopp, 1991). CD8 cells comprise about 20% of PBM. CD8 is expressed on a subpopulation of lymphocytes in the paracortex of lymph node and spleen and on about 70% thymocytes. It is not expressed on B cells, monocytes/macrophages or granulocytes. CD8 cells are rarely seen in the B dependent zones and follicular areas of lymph nodes (Ellis *et al*, 1986). In the peripheral blood, most cattle CD8<sup>+</sup> cells are CD2<sup>+</sup> and  $\alpha\beta$  TCR<sup>+</sup>, but a few cells express  $\gamma\delta$  TCR as well (Howard & Naessens, 1993). Most cattle have slightly higher levels of CD4<sup>+</sup> cells than CD8<sup>+</sup> cells in the peripheral blood (Nichani, 1995).

Bovine CD8 cells recognise antigen presented by MHC class I molecules (MacHugh and Sopp, 1991). Class I consists of a heavy chain of 44kD, comprising three extracellular domains, a transmembrane section and a cytoplasmic region (Brown *et al*, 1989). An antigen binding site is formed between the  $\alpha_1$  and  $\alpha_2$  domain. These MHC class I antigens are found on every nucleated cell. They are involved in the recognition of self and act as classical alloantigens. MHC class I present endogenously processed peptide antigens to CTL

Cytotoxic effector function resides in CD8<sup>+</sup> population in an allogeneic mixed lymphocyte culture or *in vitro* generated cytotoxic T cell lines against intracellular *T. parva* ( Goddeeris *et al*, 1986) and *T. annulata* (Innes *et al*, 1989). Most CD8<sup>+</sup> cells

do not produce IL-2 and purified CD8<sup>+</sup> cells respond very poorly to mitogens or alloantigens in the absence of exogenous growth factors (Ellis *et al*, 1986).

#### 2.3.4 CD3

This molecule consists of five different polypeptide chains with mw ranging from 12-44 kD. CD3 is present on all T cells with  $\alpha\beta$  or  $\gamma\delta$  TCR, but not on B lymphocytes, monocytes and granulocytes (Davies *et al*, 1993). The five chains are closely associated with each other and also with TCR which is essential for T cell maturation and function. Incubation of T cells with anti-CD3 antibody induces calcium flux and proliferation. This group of molecules, therefore, may be involved in transmitting signals to the cell interior following binding of antigen to TCR (Clevers *et al*, 1988).

#### 2.3.5 WC1<sup>+</sup> $\gamma\delta$ T cells

CD2<sup>+</sup> CD4<sup>-</sup>CD8<sup>-</sup>CD3<sup>+</sup> T cells in human have been reported to express another form of TCR made up of  $\gamma$  and  $\delta$  chains (Brenner *et al*, 1988) and are called WC1<sup>+</sup> cells in cattle and sheep (Morrison & Davies, 1991). A similar population of cells in the peripheral blood has also been identified in sheep (Mackay *et al*, 1988; Miyasaka *et al*, 1988). In cattle,  $\gamma\delta$ T cells are CD2<sup>-</sup> (Clevers *et al*, 1990). Northern blot hybridisation using  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$  probes showed that these cells express  $\gamma$  and  $\delta$  message (Hein *et al*, 1989; Clevers *et al*, 1990).

In humans  $\gamma\delta$ T cells constitute <5% of PBM whereas these cells form a substantial population in the peripheral blood of young cattle where they might be as high as 25% in calves of less than three weeks of age. However, this population decreases with age in peripheral blood (Clevers *et al*, 1990). Bovine  $\gamma\delta$ T cells express a surface molecule known as WC1 (Clevers *et al*, 1990) which exists in two different forms,

p215 and p205, both recognised by a mAb CC15. This molecule is exclusively expressed on CD2<sup>+</sup> CD4<sup>+</sup>CD8<sup>+</sup>  $\gamma\delta$ T cells but is, as yet, without any known function (Crocker *et al*, 1993). These cells originate in the thymus and hence belong to T cell lineage. They are mainly concentrated in the thymic medulla, but a few are scattered through thymic cortex as well. In the lymph node,  $\gamma\delta$ T cells occur in the outer areas of the cortex adjacent to the subcapsular sinuses. A few cells are also present in the sinuses and in paracortex but not in B cell follicles.  $\gamma\delta$ T cells are concentrated in the marginal zones of spleen (Clevers *et al*, 1990). They can proliferate in response to mitogens but do not produce their own growth factors. They need IL-2 to induce their proliferation (Clevers *et al*, 1990). This has been further confirmed by Collins (1993) who showed that WC1<sup>+</sup>  $\gamma\delta$ T also produce TNF $\alpha$  which may be involved in cytotoxic or cytostatic activity.

### **2.3.6 IL-2 receptor**

IL-2R consists of  $\alpha$ ,  $\beta$ , and  $\delta$  chains. Two surface molecules mediate binding of IL-2 to cells: CD25 a glycoprotein of 50kD mw (Tac antigen or IL-2R $\alpha$ ) which binds IL-2 with low affinity and a 75 kD antigen (IL-2R $\beta$ ) which binds IL-2 with a slightly higher affinity (Malek *et al*, 1983; Robb *et al*, 1987). The heterodimeric complex of both surface molecules forms a functionally effective high affinity receptor which mediates cell proliferation. Binding of IL-2 & IL-2R also leads to internalisation of the IL-2/IL-2R complex and to downregulation of IL-2R expression on the cell surface. Without continuous stimulation, functional IL-2R disappears and the activated cell returns to a resting stage (Cantrell & Smith, 1983).

Unstimulated T and B cells can express IL-2 $\alpha$  or IL-2 $\beta$  in very low quantities, but the expression of high quantities of IL-2R $\alpha$  and to a lesser extent IL-2R $\beta$ , on the cell

surface occurs only after stimulation. So far only IL-2R $\alpha$  has been characterised in cattle (Naessens *et al*, 1992). The antigen is not detected on resting PBM but is induced after activation with mitogens. IL-2R $\alpha$  is also expressed on long-term cultured T cell lines, on CD2<sup>+</sup>CD8<sup>+</sup> or CD2<sup>-</sup>CD8<sup>+</sup> T cell clones (Naessens *et al*, 1992) and most *T. parva* infected lines of T or B cell origin and some *T. annulata* infected cell lines of non T cell origin (Dobbelaere *et al*, 1990). A low level of CD25 expression is seen on resting bovine WC1<sup>+</sup> T cells in PBM (Howard & Morrison, 1994). Monocytes and B cells in stimulated bovine PBM cultures also express IL-2R $\alpha$  antigen at a lower intensity than T cells (Taylor *et al*, 1992). The mAb to bovine CD25 antigen blocks IL-2 driven proliferation even at high concentrations of IL-2 and is a useful tool for measuring the activation state of particular cells as well as for studying IL-2 dependent cell proliferation (Naessens *et al*, 1992).

### **2.3.7 Leucocyte common antigen (LCA)**

This antigen is reported as CD45R and consists of at least five high molecular weight glycoproteins present on the surface of majority of leucocytes. Different LCA isoforms arise from a single gene by alternative mRNA splicing of three individual exons potentially generating eight different molecules. Variation between all the isoforms is in the extracellular region (Thomas, 1989). The larger intracellular portion is identical in all isoforms and has protein tyrosine kinase activity. LCA can thus potentially interact with intracellular protein kinases and may be involved in triggering cell activation (Clarke & Ledbetter, 1989).

CD45R is now divided into three LCA isoforms: CD45RO, CD45RA and CD45RB. CD45RO isoform is a 180 kD molecule in humans and is expressed on memory T cells or primed T cells. CD45RA isoform is a 220 kD molecule expressed on naive

or virgin T cells (Akbar *et al*, 1988; Beverley, 1990). It is believed that a naive T cell which is CD45RA<sup>+</sup>/CD45RO<sup>-</sup> changes to CD45RA<sup>+</sup>/CD45RO<sup>+</sup> and finally to CD45RA<sup>-</sup>/CD45RO<sup>+</sup> after exposure to an antigen. The third isoform CD45RB consists of four molecules with molecular weights between 190-220 kD and is expressed on some T cells, most B cells and monocytes. Naive cells are CD45RB<sup>+</sup> (high) and memory cells are CD45RB<sup>+</sup> (low) (Streuli *et al*, 1988).

In cattle, naive CD4<sup>+</sup> cells were shown to be CD45RB<sup>+</sup> (high) and memory CD4<sup>+</sup> cells were CD45RB<sup>+</sup> (low), but MHC class I restricted CD8<sup>+</sup> cells were heterogeneous with respect to the LCA isoform they express (Howard *et al*, 1991b). CD45RO antigen was shown to be expressed on the majority of bovine monocytes, granulocytes and  $\gamma\delta$  T cells, variably expressed on CD2<sup>+</sup> T cells and absent from B cells. The ability to proliferate in response to recall antigens resided in the CD45RO<sup>+</sup> cell population amongst CD4<sup>+</sup> cell populations (Bembridge *et al*, 1993). Sheep B cells, NK cells and naive T cells expressed CD45RA and the proliferative ability to recall antigens resided within the CD45RA<sup>-</sup> population (memory) amongst CD4<sup>+</sup> cells (Mackay *et al*, 1990). T cells in afferent lymph expressed a memory phenotype, but most T cells in efferent lymph were naive T cells. The number of naive T cells in the peripheral blood decreased as the age of animals increased (Mackay, 1993).

### **2.3.8 B cells**

B cells represent about 5-15% of the circulating lymphoid pool and are classically defined by the presence of endogenously produced immunoglobulin (Ig) on the surface. Polyspecific heteroantisera against bovine Ig have been used to identify B cells for many years (Grewal *et al*, 1978). Isotype specific heteroantisera which react with IgM, IgG<sub>1</sub>, IgG<sub>2a</sub> or IgA produced in goats have also been used to identify B



cells (Nielsen *et al*, 1985). Monoclonal antibodies are now available against most of bovine Ig isotypes (Naessens *et al*, 1988; Naessens & Howard, 1991; Mukwede *et al*, 1993). The majority of peripheral blood B cells express mainly IgM antibodies and only a few express other Ig on their surface, although these are present in larger numbers under specific locations like IgA bearing cells in the gut. Subsequently, B cells may change the isotype of antibody produced, but their antigen specificity remains the same (Baldwin *et al*, 1988b).

B cells are initially activated in response to an antigen in T cell areas of the lymph node in association with interdigitating cells and T cell help. On average three activated B cells colonise a primary follicle and undergo massive clonal expansion to form secondary follicle or germinal centres. Germinal centre B cells may take up antigen held on follicular dendritic cells as an immune complex in unprocessed form, process this antigen and present it to CD4<sup>+</sup> cells (MacLennan, 1994b). The majority of B cells express MHC class II, complement and Fc receptor on their surfaces (Kunita *et al*, 1988). The expression of various antigen markers on the maturation stages of B cells, i.e. pro-B cell, pre-B cell, B cell expressing Ig, activated B cell, B cell blast and plasma cells, is not clearly defined (Mukwede *et al*, 1993).

### **2.3.9 Mononuclear phagocytic cells**

Cells of this lineage comprise functionally and morphologically heterogeneous populations. The two main functions of these cells are the phagocytosis of particulate antigens and the presentation of antigen to various subpopulations of lymphocytes. Different cells within this population occupy distinct locations within the body tissues or fluids. They include blood monocytes/macrophages, Langerhans cells of skin epidermis, Kupffer cells of liver, pulmonary alveolar macrophages, osteoclasts and

interdigitating or dendritic cells in solid lymphoid tissues (Splitter & Morrison, 1991). Differentiation of these cells in particular environments may result in the expression of certain antigens and not others. Further, antigens present on cells of this lineage may be expressed on other cell types suggesting a common functional importance of the molecules in myeloid and lymphoid cell differentiation.

### 2.3.10 Natural Killer (NK) cells

Populations of CD2<sup>+</sup> CD3<sup>-</sup> CD4<sup>-</sup> CD8<sup>-</sup> cells in bovine peripheral blood are likely to correspond to NK cells (Cook *et al*, 1989; Evans and JasoFriedmann, 1993). NK cell like activity has been shown for PBM in the killing of virus infected cells. Non-MHC restricted cytotoxicity has been well documented against bovine herpes virus (Cook *et al*, 1989; 1989a; Campos *et al*, 1992; Denis *et al*, 1993). Activation of bovine NK cells have been reported to be dependent upon IL-2 or IFN $\gamma$  (Jensen & Schultz, 1990). Cytokine mediated NK cell activity has been reported for various parasites (Akuffo *et al*, 1993; Johnson *et al*, 1993). Recently in *in vitro* studies, Campbell (1995) reported activated NK- like cells in bovine PBM after 5 days of culture with *T. annulata* infected cells. These activated NK like cells were reported to acquire a CD2<sup>+</sup> CD45RO<sup>+</sup> CD4<sup>-</sup>CD8<sup>-</sup> phenotype. To date no *in vivo* study has been conducted regarding NK like cells in *T. annulata* infection.

## 2.4 CYTOKINE MEDIATED IMMUNE RESPONSES

The role of various cytokines have been proposed in the resistance against protozoan parasite and pathogenesis of the diseases they cause. Very little is known about their role in infection with *Theileria spp*. Bovine recombinant IFN $\gamma$  (BorIFN $\gamma$ ) has been reported to significantly inhibit the *in vitro* development of trophozoite infected cells of *T. annulata* with no effect on already established macroschizont-infected cell lines.



In yet another study, PBM from the lethally infected cattle spontaneously produced IFN $\gamma$  in an advanced stage of infection (Preston *et al*, 1993). In a recent study, efferent lymph which contained high levels of IFN $\gamma$  during the first week of infection inhibited the proliferation of macroschizont-infected cell in *in vitro* assays (Nichani, 1995). Initial evidence for a role for cytokine in the generation of fever has been suggested (Preston *et al*, 1992b). In another study (Campbell, 1995), DLN cells showed the loss of IL-4 mRNA within 4 days of lethal infection with the presence of IFN $\gamma$  throughout the infection. The same study also revealed the destruction of GC with the loss of IL-4. These findings suggested the loss of IL-4 and overproduction of IFN $\gamma$  as the cause of the destruction of GC and lymph node pathology after *T. annulata* infection. High levels of IFN $\gamma$  were found in cattle undergoing severe infection (Nichani, 1995) whereas only very low levels of IFN $\gamma$  were recorded in cattle undergoing a mild reaction to immunisation with *T. annulata* cell lines (Nichani, 1995). Although no possible explanation for this low level of IFN $\gamma$  was provided, studies on other parasitic infections suggest it may be controlled by T<sub>H2</sub> cytokines (Roberts *et al*, 1996). Cytokine profiles in DLN of animals immunised with *T. annulata* have not yet been investigated.

*T. annulata* infected cell lines have been shown to produce IFN $\alpha$  (Entrican *et al*, 1991b; Preston *et al*, 1993) and IFN $\alpha$ , IFN $\beta$ , IL-6, IL-10 and TNF $\alpha$  but not IL-2 and IL-4 (Brown *et al*, 1995). The level of T cell proliferation in the autologous MLR has been correlated with the level of IL-1 $\alpha$  produced by the infected cells (Brown *et al*, 1995).

## 2.5 AIMS OF THE PROJECT

The above literature has shown immunisation of cattle with *T. annulata* infected cell line to be the widely accepted method for the control of tropical thelariosis. Previous studies have also shown the involvement of CMI in the generation of immune responses in the efferent lymph and PBM but there are no reports of T cell activation and immune responses in the draining lymph node or in the afferent lymph after immunisation with cell lines.

The work described here was divided in two main areas.

- 1) Analysis of the mechanism of activation of T cells in the draining lymph node by immunohistology and of the cytokine profiles of the draining lymph node cells by Reverse Transcription-Polymerase Chain Reaction (RT- PCR) studies.
- 2) Analysis of activation of the T cells, NK cells and the presence of IFN $\gamma$  in the afferent lymph draining the site of immunisation and investigation into the site of parasite transfer by cannulation studies.

## **CHAPTER 3**

### **RESPONSES IN THE DRAINING LYMPH NODE FOLLOWING IMMUNISATION WITH *THEILERIA ANNULATA* INFECTED CELL LINES.**

### 3.1 INTRODUCTION

Cattle can be immunised successfully against tropical theileriosis by inoculation with *T. annulata* (macroschizont) infected cell lines (cell lines). Such cell lines can be initiated *in vitro* by incubating sporozoites with uninfected bovine PBM (Brown, 1987) and can be maintained *in vitro* in an appropriate cell culture medium for an indefinite period without the addition of any growth factors (Hulliger *et al*, 1964). The virulence of these cell lines can be attenuated by prolonged *in vitro* culture (Tsur & Adler, 1962; Pipano & Israel, 1971; Pipano *et al*, 1973). Both actively growing cell lines and lines resuscitated after cryopreservation can be used as vaccines.

Animals can be immunised with  $10^2$  (Ouhelli *et al*, 1989; Innes *et al*, 1989a) or even 10 *T. annulata* infected allogeneic cells (Brown 1990). Animals which have been immunised with a cell line withstand natural or experimental challenge with sporozoites while showing mild parasitological reactions (Pipano, 1981; Ouhelli *et al*, 1989; Nichani, 1995).

The immunised animals produce cytotoxic responses as CTLs which recognise the parasitised cells expressing autologous MHC class 1 molecules (Preston *et al*, 1983; Innes *et al*, 1989a). In a recent report from this laboratory, Nichani (1995) recorded the presence of anti-allogeneic and anti-parasite CTLs in the efferent lymph from the draining lymph node (DLN) in a calf immunised with  $10^6$  cells of an allogeneic *T. annulata* (Hissar) infected cell line. This report (Nichani, 1995) suggested that immune responses to the cell lines were initiated in the lymph node draining the site of immunisation with cell lines.

High antibody titres have been reported in cattle after immunisation with attenuated

*T. annulata* cell lines (Shukla and Sharma, 1989; Kachani 1992a, 1992b). Although sera from immune cattle recognised the parasite inside the host cells as assessed by IFAT, transfer of such sera to susceptible animals did not protect them from infection (Dhar & Gautam, 1978; Samad *et al*, 1984). The role that these antibodies play *in vivo* is still unknown.

In the absence of reinfection, the duration of immunity after immunisation with cell lines has not been fully investigated. However, it was reported to be 18 months (Pipano, 1977) or a year (Singh, 1992; Singh *et al*, 1993) in cattle immunised with attenuated cell line vaccines. Zablotzky (1991) reported that immunity can last for as long as 3.5 years after immunisation. In a recent report, animals were susceptible to sporozoite challenge as early as 7 months after immunisation with *T. annulata* infected cell line (Ouhelli *et al*, 1994).

*T. annulata* macroschizont infected cells induce nonspecific proliferation of resting PBM (Glass *et al*, 1990a). Similar findings have been suggested to occur in the DLN after sporozoite infection (Campbell, 1995). Activation at abnormal sites namely the medulla has been reported (Campbell, 1995) in animals lethally infected with sporozoites and may be considered as one of the main reasons for the failure of the immune response in such animals.

As far as we know, there is no report in the literature about the immune responses in the DLN of cattle after immunisation with *T. annulata* cell lines including how macroschizonts activate T cells. The study in this chapter was undertaken with the aim of understanding the mechanisms of T cell activation involved in the generation of immune responses in the DLN during *T. annulata* cell line immunisation.

### 3.2 EXPERIMENTAL DESIGN

The cell line as used by Nichani, at the same cell concentration was used in the experiments described in this thesis. Ten young calves were BoLA typed for their MHC class I. Four calves with MHC class I different to that of the immunising cell line were selected for this experiment. The selected calves were immunised with  $10^6$  cells of the allogeneic *T. annulata* infected cell line to provide draining lymph nodes for examination by immunohistology.

This experiment was conducted in two batches. Initially two young calves were immunised and their prescapular lymph nodes were removed at days 4 and 12 after immunisation. Two more calves were then immunised and their lymph nodes were removed at days 9 and 16 (Table 3.1). All the lymph nodes were studied in detail for their immune responses by histological and immunohistological techniques.

**Table 3.1**

#### **Experimental design**

<b>Animal number</b>	<b>Cell line</b>	<b>Day at which DLN removed</b>
13046	<i>T. annulata</i> (Hissar) 10769	Naive (Unimmunised)
13054	<i>T. annulata</i> (Hissar) 10769	Naive (Unimmunised)
12848	<i>T. annulata</i> (Hissar) 10769	4
12810	<i>T. annulata</i> (Hissar) 10769	9
12851	<i>T. annulata</i> (Hissar) 10769	12
12852	<i>T. annulata</i> (Hissar) 10769	16



Young calves (2 months old) were chosen because of the minimum chances of their exposure to environmental antigens. Calves younger than this age could not be used as they are usually not weaned until one and a half months of age.

A cell dose of  $10^6$  was chosen because it has shown that the animals immunised with this dose are well protected upon lethal challenge (Shukla *et al*, 1989; Innes *et al*, 1989a; Nichani *et al*, 1995).

An allogeneic cell line was chosen because of its practical benefits in field vaccination. In field vaccination programmes, it is not possible to immunise the animals with an autologous or BoLA matched cell line. Moreover, using allogeneic cell lines are much safer than using autologous cell lines (Innes *et al*, 1989a).

### **3.3 MATERIALS AND METHODS**

#### **3.3.1 Animals**

The animals used in this study were Friesian (British) or Ayrshire calves aged about 2 months at the start of experiments. All calves were selected after BoLA typing and were immunised with  $10^6$  cells of the *T. annulata* (Hisar) 10769 infected cell line. The cell line was injected subcutaneously above the prescapular lymph node in the neck region. They were left in the animal shed after immunisation. Their prescapular lymph nodes were removed under general anaesthesia at days 4, 9, 12 and 16 after immunisation.

#### **3.3.2 MHC class 1 typing of animals (BoLA typing)**

The microlymphocytotoxicity test described by Spooner *et al*, (1979) was performed to identify the MHC class 1 type (BoLA type) of the calves. The MHC Class 1 type

of the *T. annulata* infected cell lines isolated from the immunised animals was also determined by the microlymphocytotoxicity test as described by Spooner & Brown (1980).

#### **3.3.2.1 Panel of alloantisera for BoLA typing**

The panel of alloantisera used to define the BoLA specificities of the animals and the *T. annulata* infected cell lines used in this study were produced in this laboratory from cattle by reciprocal skin grafting between dam and offspring (Spooner *et al*, 1979). These operationally monospecific sera have been tested and compared with sera from other laboratories in the five international comparison workshops carried out to date (Spooner *et al*, 1979; Anon, 1982; Bull *et al*, 1989; Bernoco *et al*, 1991; Davis *et al*, 1994a) and have been assigned workshop (w) specificities.

The BoLA typing sera were stored neat in small aliquots at -70°C. Control serum was selected for non reactivity with any of the test cells and was used in the lymphocytotoxicity test to estimate baseline test cell viability. The antibodies were coated onto Terasaki plates, which were stored at -70°C.

#### **3.3.2.2 Preparation of test cells**

Lymphocytes for the microlymphocytotoxicity test were prepared as described by Spooner *et al*, (1979). Three ml of venous blood was collected in a sterile vacutainer containing lithium heparin (Becton Dickinson). The blood was layered onto 2.5ml of Ficoll-hypaque solution of specific gravity (S.G.) 1.077 (Lymphoprep) (Nycomed Pharma As, Oslo, Norway) and was centrifuged for 15 minutes (min.) at 1500g. The resultant lymphocyte layer was collected at the plasma/Ficoll interface and washed in Hank's balanced salt solution (HBSS) by centrifugation at 400g for 5 min. The cell

pellet was resuspended in small volume of HBSS and any contaminating RBCs were lysed by adding 2ml of deionised distilled water to the cell sample with constant mixing, rapidly followed by addition of 2ml of double strength HBSS. The cell suspension was washed twice with HBSS by centrifugation at 100g for 5 min. (to remove platelets) and resuspended in HBSS. Cells were counted in a haemocytometer (Neubauer chamber) using a phase contrast microscope (Diavert, Leitz) and adjusted to give a final cell concentration of  $2 \times 10^6$  per ml in HBSS for testing.

### 3.3.2.3 *Microlymphocytotoxicity test*

This test was conducted as described by Spooner *et al*, (1979). The Terasaki typing plates, on which the panel of antibodies had been coated, were thawed prior to use and kept at room temperature. The test cell suspension (1 $\mu$ l) was added to each well and the plates were incubated for 30 min. The complement (5 $\mu$ l) was added to each well and further incubated for 1 hour (hr). Two  $\mu$ l of 5% eosin dye was added to each well and incubated for 5 min. Finally, 5 $\mu$ l of fixing solution (see appendix) was added. Plates were then read immediately using an inverted phase contrast microscope (Nikon) at 100x magnification or stored at -4<sup>0</sup>C and read within a week. Test plates were scored as in Table 3.2 using the system described in the second international BoLA workshop (Anon, 1982).

**Table 3.2****Scoring of Terasaki plates**

Score	% killed cells
8	80-100
6	60-79
4	30-59
2	10-29
1	0-9

**3.3.3 *Theileria annulata* macroschizont-infected cell lines and sporozoite**

The stocks of *T. annulata* used in this study are detailed in the relevant chapters. The majority of the work involved a *T. annulata* cell line 10769 infected with the Hissar stock of *T.annulata* (Gill *et al.*, 1980). Parasite material used included cell lines, ground up tick supernatant (GUTS) for establishing cell lines and cryopreserved tick stabilates for challenging animals.

**3.3.3.1 *In vitro* derived *Theileria annulata* infected cell lines**

*T. annulata* infected cell lines were established *in vitro* as described by Brown (1983). The technique, which involved incubating bovine peripheral blood mononuclear cells (PBM) with sporozoites prior to establishing the infected cell lines *in vitro*, is briefly described here. The cell lines were maintained in complete tissue culture medium (TC).

#### 3.3.3.1.1 *Isolation of peripheral blood mononuclear cells (PBM)*

Venous blood was collected in vacutainers containing ACD. PBM were separated by centrifugation over Lymphoprep at 1500g for 25 min. at room temp. PBM were removed from the plasma/Ficoll interface and washed in phosphate buffer saline (PBS) by centrifugation at 300g for 10 min. The cells were further washed twice in PBS and once in RMPI-1640 by centrifugation at 100g for 10 min., resuspended in complete tissue culture medium, counted and adjusted to the required cell concentration.

#### 3.3.3.1.2 *Preparation of ground up tick supernatant (GUTS)*

Tick material was prepared and stored by the staff at the Centre for Tropical Veterinary Medicine, Edinburgh and kindly provided as and when required. The procedure for obtaining a sterile preparation of GUTS as a source of *Theileria* sporozoite material (Brown, 1983) is described briefly below.

Adult *Hyalomma anatolicum anatolicum* ticks infected with *T. annulata* (Walker *et al*, 1985) were fed for 3 days on a rabbit's ears to stimulate maturation of the sporozoite. The engorged ticks were then removed and washed once in 1% benzalkonium chloride (Roccal, Winthrop) and a further 3 times in 70% ethanol. They were then transferred to a sterile container and washed 3 times in warm Eagles minimal essential medium (MEM) with Hanks salts, double strength antibiotics (penicillin, 200i.u. per ml and streptomycin 0.2mg per ml) (Gibco, BRL) and nystatin 0.1mg per ml (Gibco, BRL). They were left for ten min. in the fourth wash. The medium was discarded, and 2-5 ml of cold MEM with 3.5% bovine plasma albumin (BPA) (Fraction V, Sigma ) containing antibiotics as described above was added to the ticks, which were then transferred to a sterile mortar. They were then ground hard

with a sterile pestle, with an aliquot of supernatant being removed, and measured quantities of MEM and 3.5% BPA solution added. This was repeated until the required concentration of supernatant material (Measured as tick equivalents per ml, usually 4 t.e. per ml ) was reached.

The supernatant was centrifuged for 5 min. at 100g, removed and filtered through a sterile 25mm or 47mm Millipore Swinnex filter with an AP prefilter and 8  $\mu$  MF filter (Millipore Corporation). The resultant filtrate consists of sterile GUTS.

#### **3.3.3.1.3 *In vitro* infection of bovine PBM with *Theileria annulata* sporozoites**

This technique, described in detail by Brown (1983), may use either fresh GUTS filtrate or the cryopreserved GUTS. In the first case,  $2 \times 10^7$  PBM in 1 ml TC medium were mixed with 1 ml of the fresh sporozoite suspension (GUTS filtrate) at 1.0 t.e. per ml in a 25 cm<sup>2</sup> tissue culture flask (Intermed, Nunc). The flask was kept upright in a CO<sub>2</sub> incubator at 37°C for two hours. After 2 hrs, 8 ml of TC medium was added to the flask and the flask was placed in the normal horizontal position in an incubator.

In the second case, cryopreserved GUTS filtrate was rapidly thawed immediately after removal from storage in liquid nitrogen, kept at room temp. for 20 min. and serially diluted slowly with TC medium containing 20% foetal calf serum (FCS). It was diluted 3 times with a double dilution at 20 min. intervals. The diluted tick material (8 ml) was then mixed with 2 ml of  $1 \times 10^7$  PBM per ml in a 25 cm<sup>2</sup> flask. The flask was kept in an upright position overnight in a 5% CO<sub>2</sub> incubator at 37° C. The next day, 7 ml of supernate was removed gently from the flask and replaced with 7 ml fresh TC medium. The flask was then placed horizontally in the incubator.

The cultures were incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator. The TC medium was changed twice a week according to metabolic status of the cultures. Once 30% or more cells were infected, the cultures were transferred to a larger culture flask and the cells were then usually maintained at a concentration between 10<sup>6</sup> to 2x10<sup>6</sup> per ml.

#### **3.3.3.2 *In vivo* derived *T. annulata* infected cell lines**

Parasite infected *in vivo* cell lines were established from the immunised animals according to the method described by Brown (1987). PBM from the blood were adjusted to 2x10<sup>6</sup> cell per ml and seeded at 2 ml per well in 24 well plates. Cultures were monitored for growth of infected cells by making smears on a cytocentrifuge.

#### **3.3.4 Cryopreservation and resuscitation of parasite material**

Both parasite infected cell lines and GUTS were cryopreserved according to the technique described by Brown (1983). 10% dimethyl sulphoxide (DMSO) (BDH) was used to cryopreserve the infected cell lines and cells. Cells were resuspended in 50% FCS in RPMI at a concentration (conc.) of 2x10<sup>7</sup> cell per ml. 20% DMSO in FCS was added slowly to the cells. The cells were then aliquoted in 1ml and stored overnight at -70°C prior to freezing in liquid nitrogen. The GUTS were cryopreserved using 7.5% glycerol (BDH) as cryoprotectant in MEM supplemented with 3.5% BPA and stored in liquid nitrogen

Cell lines were resuscitated as described by Brown (1987). The frozen cells were thawed rapidly to 37°C by keeping the vials in a water bath. Warm TC medium was added to the cells to dilute the DMSO. DMSO was removed after centrifugation of cells. The cells were resuspended in TC medium and incubated at 37°C in 5% CO<sub>2</sub>

incubator.

The sporozoite stabilates were also thawed at 37°C and left at room temperature for about 20 min. They were used within an hour after thawing.

### **3.3.5 Monitoring of animals**

#### **3.3.5.1 *Clinical observations***

The clinical condition of the calves was monitored by observing their general condition, eg. feeding, and recording rectal temperatures. A temperature of  $\geq 39.5^{\circ}\text{C}$  was considered as a febrile reaction. Any enlargement of lymph nodes and changes in colour of mucous membranes were also recorded.

#### **3.3.5.2 *Haematological observations***

Venous blood was collected in 2ml vacutainers containing disodium EDTA (Becton Dickinson) for haematological examinations. Packed cell volume (PCV), total erythrocyte count (TEC) and total leucocyte count (TLC) were measured usually twice a week after immunisation. PCV was estimated by a microhaematocrit technique. Blood was drawn into microhaematocrit tubes and the one end was sealed with plasticine, tubes were centrifuged at 12,000 rpm for 15 min. in a microhaematocrit centrifuge (Biofuge, Heraeus Sepatech). PCV was determined by a microhaematocrit reader. TEC and TLC were measured by a coulter counter (Coulter ZBI electronic particle counter, Coulter Electronics Ltd.).

#### **3.3.5.3 *Parasitological observations***

Blood smears were examined for evidence of the parasite in immunised animals. Smears were prepared by spreading a small drop of blood onto a clear glass slide



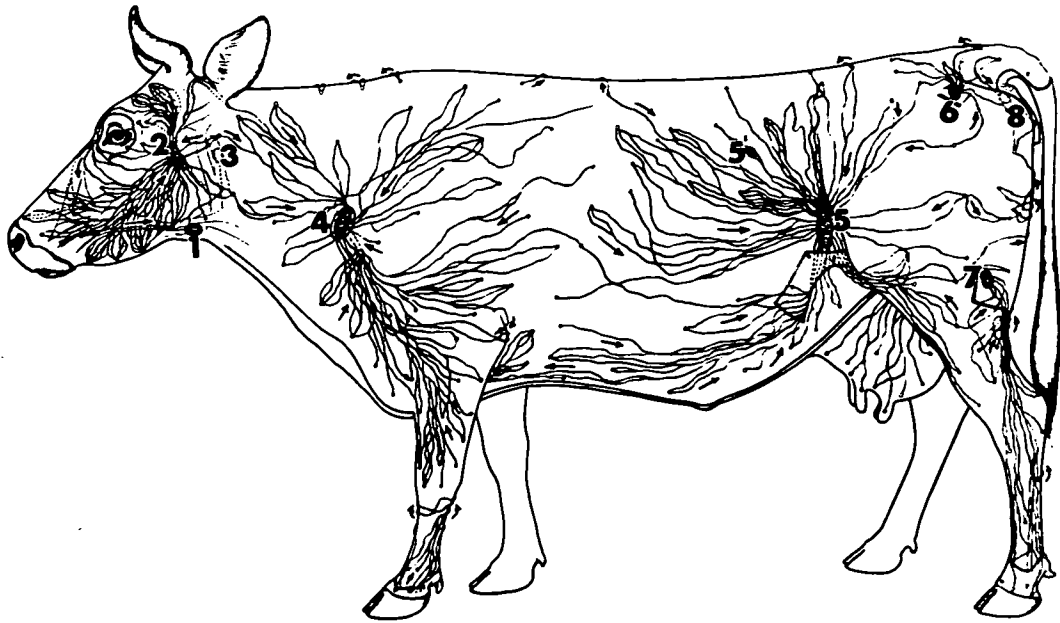
using the edge of a second slide followed by rapid air drying. The smears were fixed in methanol for 5 min. and stained with Giemsa stain (1:10 dilution in Gurr buffer) for 45 min. The slides were then rinsed in Gurr buffer and dried prior to microscopical examination.

### **3.3.6 Removal of lymph nodes for histology**

The anatomical location of bovine prescapular lymph node is shown in Fig. 3.1 (Adapted from "Sisson and Grossmans' the anatomy of the domestic animals" by Getty, 1975). All surgical operations were carried out by Dr. Roger L. Spooner.

Calves were starved for a day before anaesthesia. They were anaesthetised by intravenous (i/v) injection of 10% thiopentone sodium (Interval sodium, Rhone Merieux, Ireland.) at 1ml per 10kg body weight and intubated immediately using a cuffed endotracheal tube. Anaesthesia was maintained by a mixture of halothane, nitrous oxide and oxygen using a closed circuit anaesthetic machine fitted with a CO<sub>2</sub> absorber and rebreathing bag. The area around the prescapular lymph node was clipped and scrubbed. The site was sprayed with 5% hibitane to disinfect the area.

The prescapular lymph node was palpated and an incision was made just above it parallel to the jugular vein. A thin layer of the brachiocephalic muscle was divided by blunt dissection and held open with retractors. Blood vessels were identified and tied off. The lymph node was lifted slightly using the fingers and the blood vessels cut off. The lymphatic vessels were cut in such a way as to leave the cut end of the efferent duct open to facilitate anastomosis with the afferent. The muscles and skin were repaired using continuous sutures (Mersilk 2/0, W193, Ethicon) for the muscle and mattress sutures (Supramid, Melsungen AG) for the skin.



**Superficial lymph flow of the cow.**

1. Mandibular ln.; 2. parotid ln.; 3. lateral retropharyngeal ln.; 4. superficial cervical ln.; 5. subiliac ln.; 5'. Inn. of para-lumbar fossa; 6. gluteal ln.; 7. popliteal ln.; 8. tuberal ln. (After Baum, 1912.)

Fig. 3.1 Position and areas drained by various superficial lymph nodes. Note the position of prescapular node (4). (Figure adapted from "Sisson and Grossmann's The anatomy of domestic animals". Editors R. Getty, W.B. Saunders Co., 1975).

Animals were moved to a recovery area and allowed to recover. Clinical, haematological and parasitological observations were recorded.

**3.3.7 Processing of lymph node for histological examination**

**3.3.7.1 Paraffin embedding of lymph nodes**

As soon as the lymph nodes were extirpated from the animals, fat was removed using sterile scalpels. They were cut into approximately 1cm<sup>2</sup> pieces and fixed in 10% paraformaldehyde (Sigma) in PBS pH 7.6 for 48 hrs. These pieces were then enclosed in the tissue embedding cassettes and transferred to a vacuum enclosed tissue processor timed as follows:

70% alcohol	2hrs	
90% alcohol	2hrs	
100% alcohol	1hr	4 changes
Xylene	1hr	2 changes
Paraffin wax	1hr	4 changes

The nodes were subsequently embedded and blocked in 57<sup>0</sup>C melting point paraffin wax using a Shandon VIP automatic tissue embedder. Blocks were then kept at room temperature before cutting the sections.

2.5-3 µm sections were cut from the finished blocks using a Leitz microtome. The sections were floated on a 50<sup>0</sup> C water bath and collected upon poly-L-lysine (Sigma) coated slides and dried overnight at 55<sup>0</sup>C. They were then kept at room temperature until used.

**3.3.7.2 Staining with Haematoxylin and Eosin**

The sections were dewaxed in 2 washes of xylene, and rehydrated through graded

alcohols : 100%/95%/70%/50% absolute alcohol/distilled H<sub>2</sub>O. They were washed in running tap water before staining in haematoxylin for 3 minutes (Harris' Haematoxylin, BDH). After washing in running tap water, sections were kept in Scott's Tap Water (Saturated lithium carbonate solution) for about 1 min. to stain nuclei blue. They were then stained with Eosin solution for 30 seconds, washed in running water, dehydrated through graded alcohol, cleared in xylene and mounted in DPX mounting medium (BDH).

### 3.3.7.3 Immunohistological techniques for staining with monoclonal antibodies

Optimal concentrations of monoclonal antibodies (mAbs) were determined. Culture supernate were used in the range of neat to 1:5 dilutions and ascites in the range of 1:10 to 1:100 dilutions.

Polyclonal rabbit anti-human CD3 has been identified as reacting with bovine tissues (Ramos-Vara *et al*, 1994). 1C7 recognises *T. annulata* schizonts (Shiels *et al*, 1986b) and has been used in the examination of infected lymph nodes (Eisler, 1988; Campbell, 1995). MIB-1 recognises a Ki-67 proliferating antigen (Gerdes *et al*, 1984) and has been of particular use in staining paraffin sections (Key *et al*, 1993, Campbell, 1995).

IL-A15 (CD11b) and IL-A111(IL-2R) required the digestion of sections with trypsin for 20 min. at 37°C before staining. Anti CD3 (A-452, Dako) required the sections to be predigested with pronase for 20 min. at room temperature. MIB-1 required microwave treatment. The sections were placed in the excess citrate buffer and microwaved on full power for 3x5 min. and allowed to cool before washing in running tap water. Staining of paraffin sections was performed according to standard

immunohistochemical techniques. Tris-HCL (TBS) was used as buffer throughout the study.

Sections were marked for identification with a diamond pen on one side of the slide. They were then dewaxed in xylene, rehydrated through the graded alcohols and washed in running tap water as for conventional staining, followed by a wash in TBS for 5 min. Wherever diaminobenzidine (DAB) was to be used as substrate, endogenous peroxidase activity was blocked by incubating the sections in 0.3%  $\text{H}_2\text{O}_2$  in methanol (1ml 30%  $\text{H}_2\text{O}_2$  in 100ml methanol) for 20 min, washing in running tap water and then in TBS for 5 min. After the TBS wash, a circle was drawn around the section with a paraffin pen (Pep pen, Dako). All sections were blocked in 20% normal rabbit serum (NRS) in TBS for 10 min. The sections were incubated with the mAb, diluted as appropriate in 20% NRS, at room temperature for 30 min. Excess fluid was then tipped off, the sections rinsed in running tap water and washed 3 times in TBS for 5 min. Sections were then incubated with biotinylated rabbit anti mouse (RAM) Ig (Dako, Glostrup, Denmark)- secondary antibody (1:400 in 20% NRS) at room temperature for 30 min. and then washed 3 times with TBS. Staining was demonstrated by the avidin-biotin complex system (ABC system, Dako). Two AB complexes were used: ABC-AP (alkaline phosphatase) and ABC-HRP (horse radish peroxidase). AB Complex was prepared by mixing one drop of avidin and one drop of biotinylated enzyme (AP or HRP) with 5 ml buffer (50mM Tris-HCL) at least 30 min. before use. The AB Complex was then added to the sections and incubated for 30 min. After washing as above, the sections were incubated for 15 min. in the dark with one of the two substrate: Vector red (Vector labs, Peterborough, UK) or DAB (Sigma) prepared just before use. DAB was used with the sections which had been incubated with ABC-HRP and Vector red was used with ABC-AP. After incubating

with the substrate the sections were washed in running tap water. Sections were counterstained in haematoxylin and rehydrated, cleared and mounted as for conventional histology. All incubations were carried in a humidified chamber at room temperature.

For staining with anti human CD3, the normal blocking serum was goat serum and the secondary antibody was biotinylated goat anti-rabbit Ig. Sections were incubated overnight at 4°C with primary mAb, in a humidified chamber.

For double staining, the sections were stained first with the mAb as for single staining but not counterstained with haematoxylin. Instead, they were washed in TBS and blocked in blocking serum and then stained with the second mAb following the same protocol as for single staining. For double staining with CD3, CD3 staining was performed first.

The sections were examined using a microscope (Nikon-Microphoto-SA) equipped with filters (Colour and H&E). They were photographed using an automatic exposure photographic unit on 64T ASA film (Kodak).

### 3.4 RESULTS

#### 3.4.1 Clinical observations

##### 3.4.1.1 *General outlook, food and water intake*

All the calves were quite normal as far as their food and water intake was concerned. The animals were slightly dull during the time of febrile reactions but were never off their food and water.

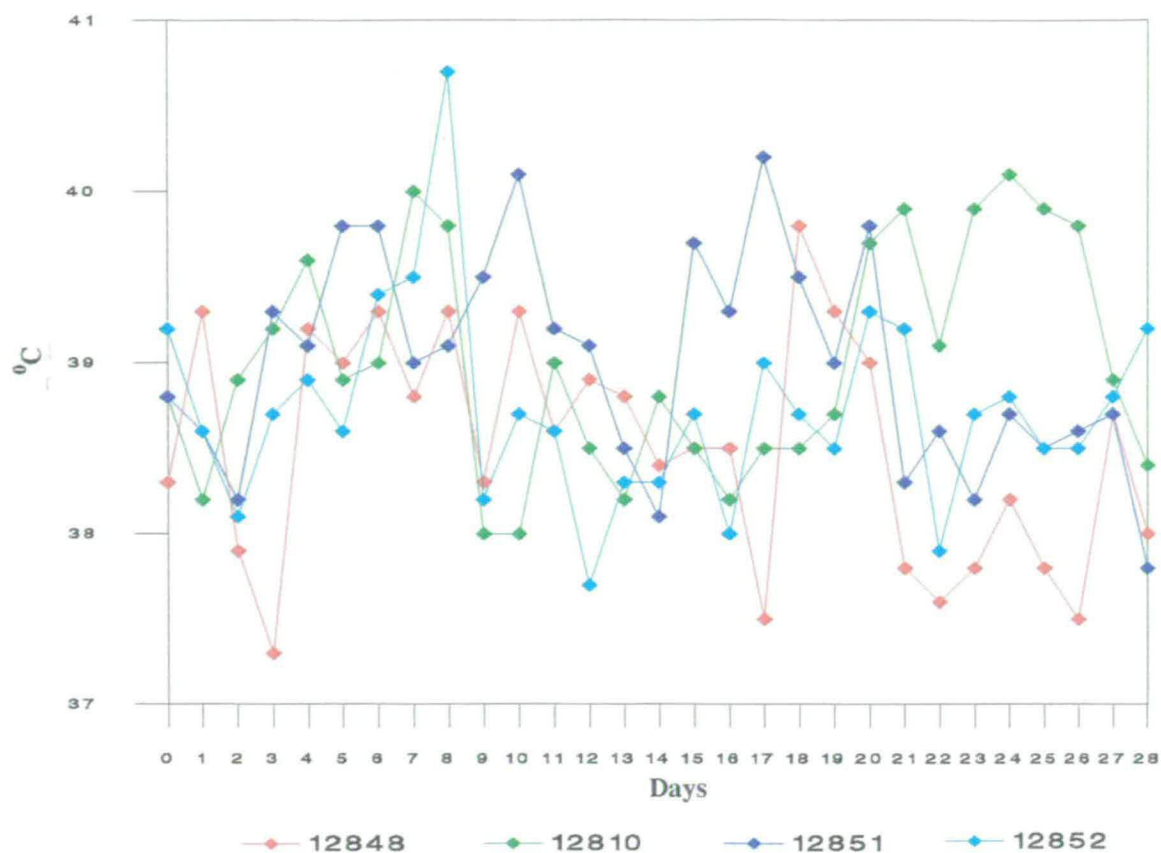
##### 3.4.1.2 *Temperature*

The first temperature (temp.) rise in calf 12848 (lymph node removed on day 4) occurred on the day the node was removed when it rose to 39.2°C (Fig. 3.2) (39.5°C and above was considered as febrile reaction). The second peak of temp., 39.8°C, was observed on day 18. Calf 12810 (node removed on day 9) showed a febrile reaction of 40°C on day 7 followed by a second rise between days 20 & 26 with a peak temperature of 40.1°C on day 24 (Fig. 3.2). Calf 12851 (node removed on day 12) underwent a febrile reaction between days 5 & 10 (peak of 40.1°C on day 10) and again between days 15 & 20 (peak of 40.2°C on day 17) (Fig. 3.2). Calf 12852 (node removed on day 16) showed a maximum temp. of 40.7°C on day 8. There was no febrile reaction after day 8 although 39.3°C was recorded on day 20 after immunisation (Fig. 3.2).

##### 3.4.1.3 *Haematological observations*

A fall in total leucocyte count (TLC) from  $9 \times 10^3/\text{cm}^2$  to  $7 \times 10^3/\text{cm}^2$  was observed on day 8 after immunisation in calf 12848. Although the normal level was regained by day 11, a second fall in TLC occurred (Fig. 3.3) in the second week after immunisation. Calf 12810 showed a fall in TLC from  $10.8 \times 10^3/\text{cm}^2$  to  $6.55 \times 10^3/\text{cm}^2$  on day 7. Calf 12851 showed a fall from  $10.65 \times 10^3/\text{cm}^2$  to  $7.9 \times 10^3/\text{cm}^2$  by day 8 with

a) Temperature



b) Haematocrit

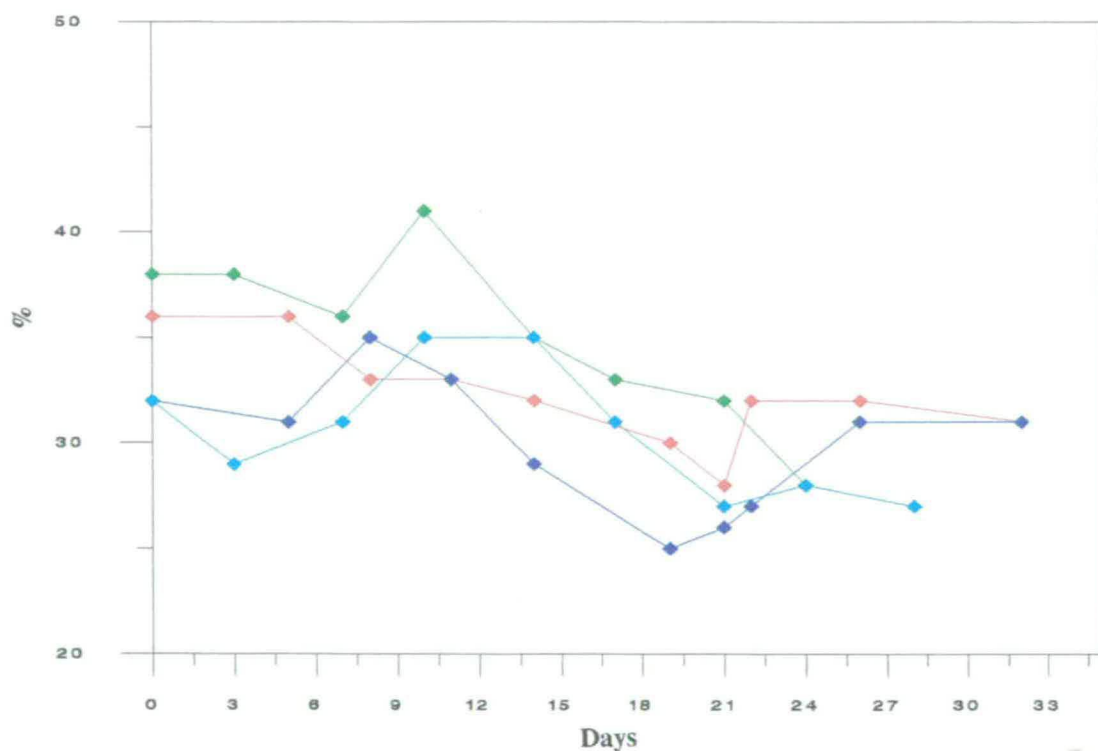
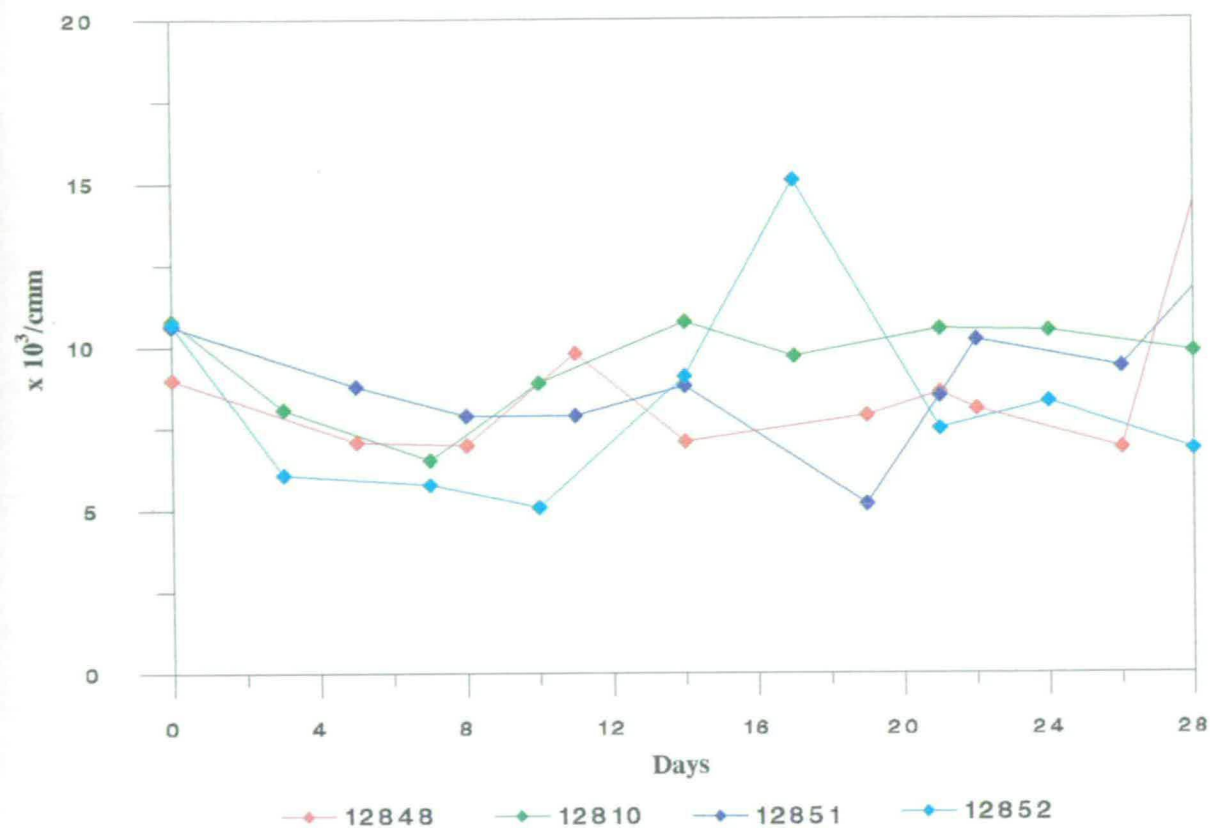


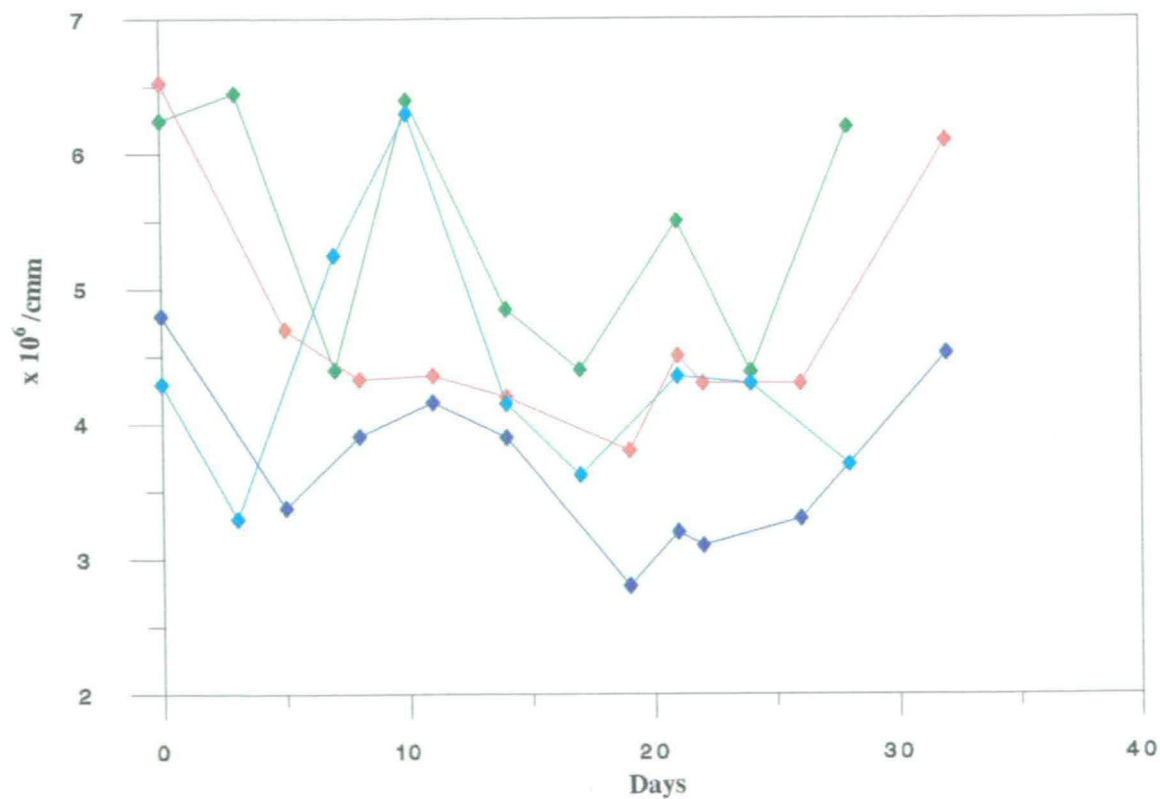
Fig. 3.2 a) Temperature and b) haematocrit of calves 12848,12810, 12851 and 12852 following immunisation with *T. annulata*(10769) cell line.



a) TLC



b) TEC



**Fig. 3.3 a) Total leucocyte count (TLC) and b) total erythrocyte count (TEC) of calves 12848,12810, 12851 and 12852 following immunisation with *T. annulata* (10769) cell line.**

a further sharp decline to  $5.2 \times 10^3/\text{cm}^2$  by day 19. Calf 12852 showed a sharp decline by day 10 when it was only  $5.1 \times 10^3/\text{cm}^2$ .

There was a sharp decline in total erythrocyte count (TEC) in calf 12848 on the days 8 and 19, in calf 12810 on days 7 and 17 and in calf 12851 on days 5 and 19 but calf 12852 showed a slight fall in TEC on days 3 and 17 (Fig. 3.3). A drop in haematocrit level in all the four calves occurred during the third week following immunisation (Fig. 3.2). Leucopenia was observed in all the calves during the first few weeks after immunisation.

### **3.4.2 Parasitological observations**

Macroschizont-infected cells were observed in cytocentrifuge preparation of PBM of all the 4 calves three weeks after immunisation. A piroplasm parasitaemia of only one to two percent was detected during the third week following immunisation in all four calves.

### **3.4.3 Morphological responses in the lymph node draining the site of immunisation as examined by histological techniques**

#### **3.4.3.1 Responses in the primary follicle and germinal centre**

A few primary follicles (PF) were observed in the lymph node of the naive animal (Fig. 3.4 ). The follicles started to take a proper shape by day 4 after immunisation (calf 12848) with a separate visible area within the cortex but without dark, light and mantle zones (Fig. 3.5). By day 9 after immunisation (calf 12810), light and dark zones appeared within small germinal centres (GC) but not surrounded by a mantle zone (Fig. 3.6). GCs became bigger in size than on day 4. By day 12 (calf 12851), the PFs changed to GC with the appearance of clearly differentiated dark and light

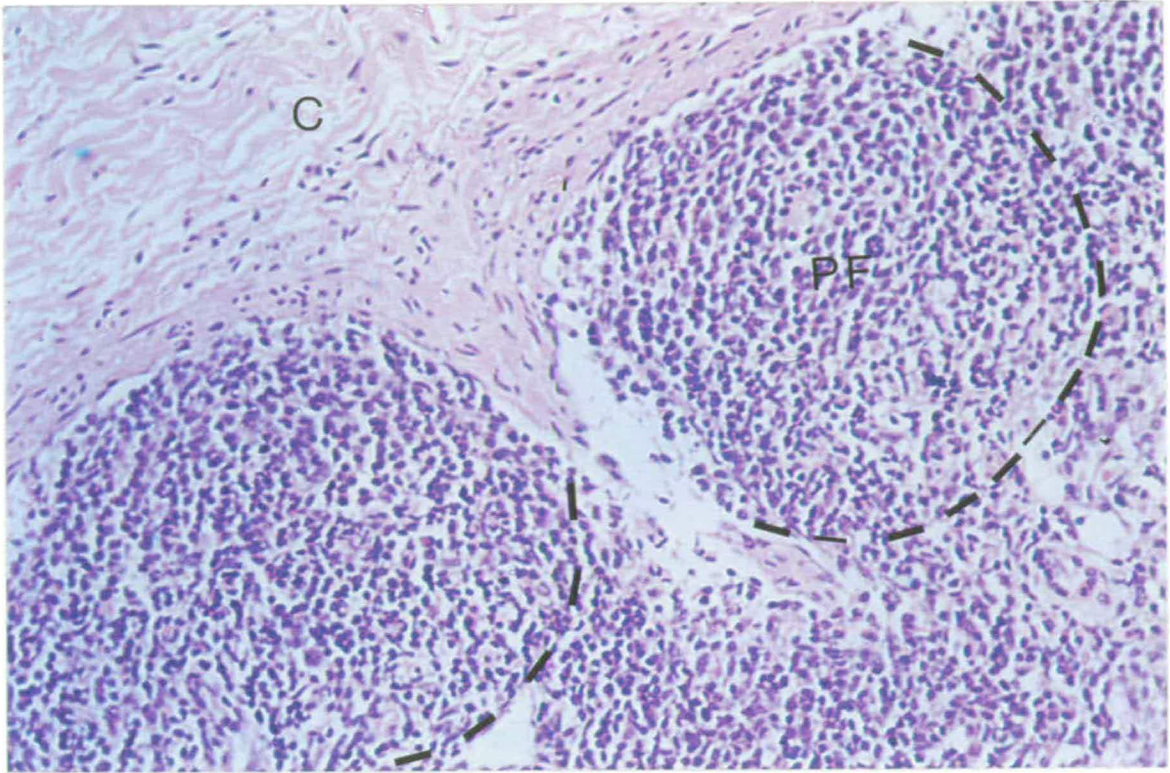


Fig. 3.4 Primary follicles (PF) in a normal lymph node. Note the outer boundary which has been marked to differentiate it from the paracortex. Capsule (C). H+E, x125.

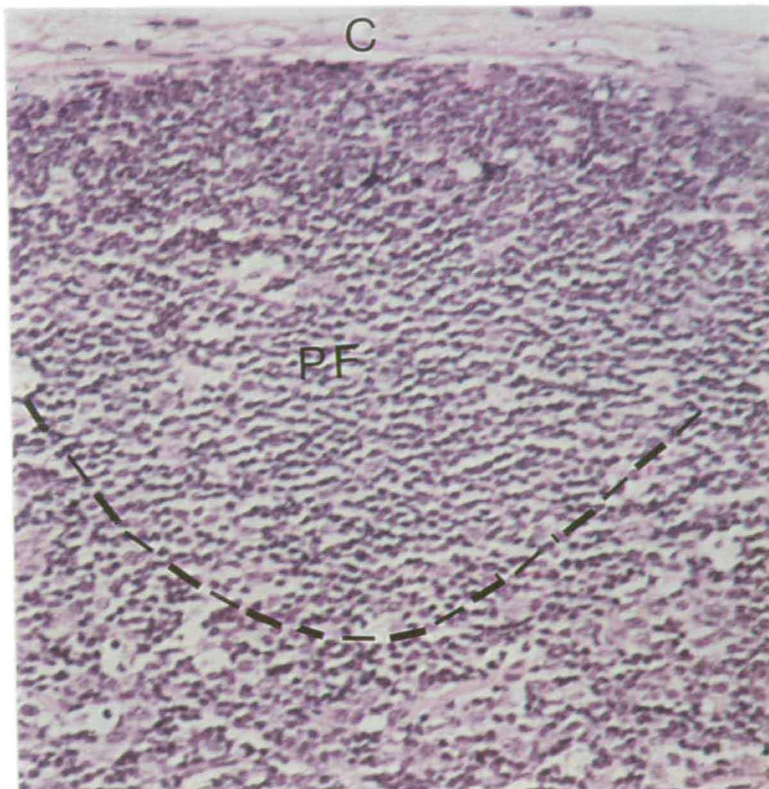


Fig. 3.5 Primary follicles (PF) in a lymph node 4 days after immunisation. H+E, x125.



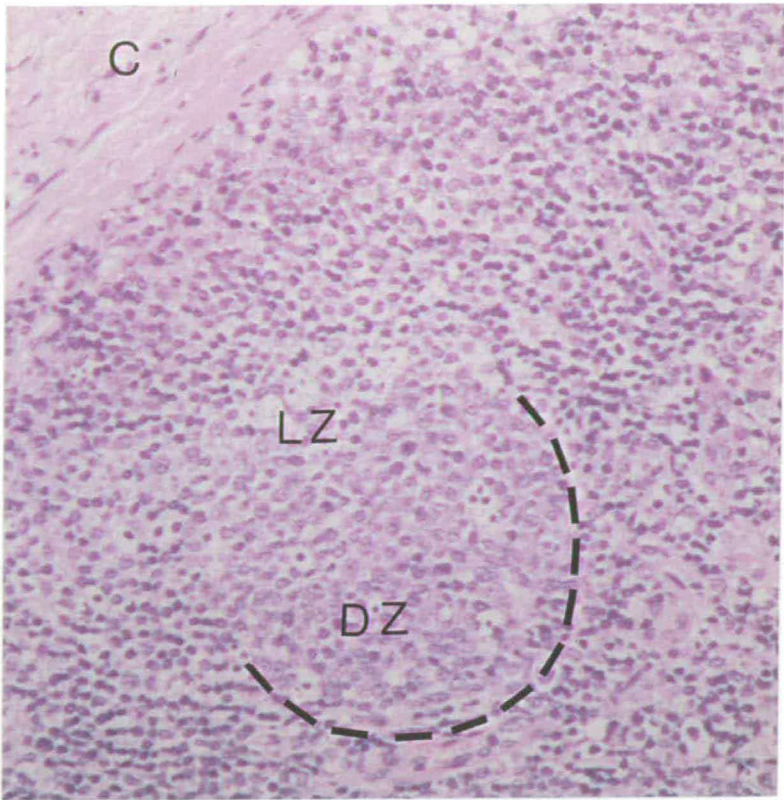


Fig. 3.6 Germinal centres in a draining lymph node 9 days after immunisation. See lower dark zone (DZ) and upper light zone (LZ) with a few apoptotic cells (†). H+E, x125.

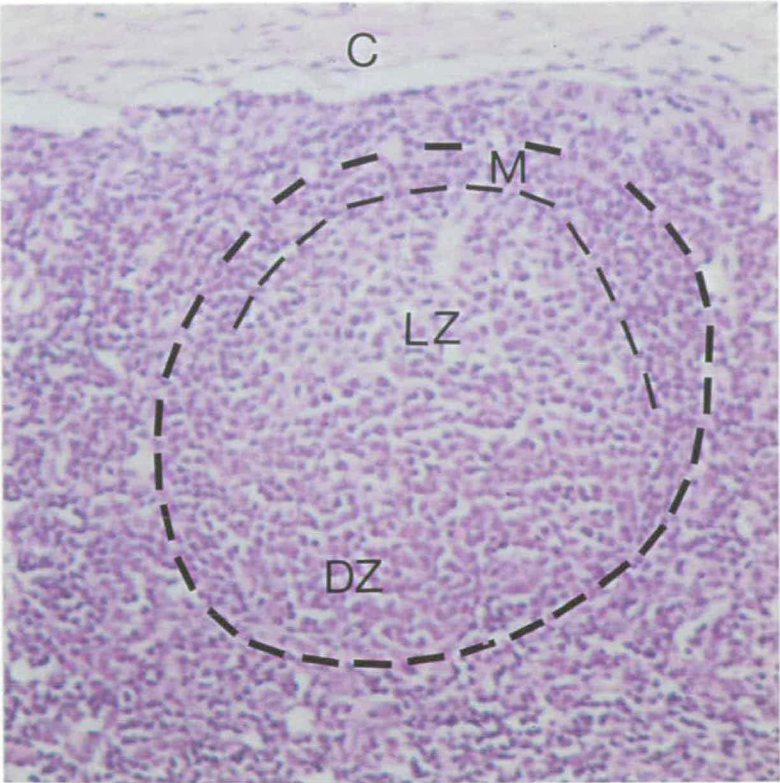


Fig. 3.7 Germinal centres with dark (DZ) and light zones (LZ) surrounded by a small mantle zone (M) in a draining node 12 days after immunisation. H+E, x125

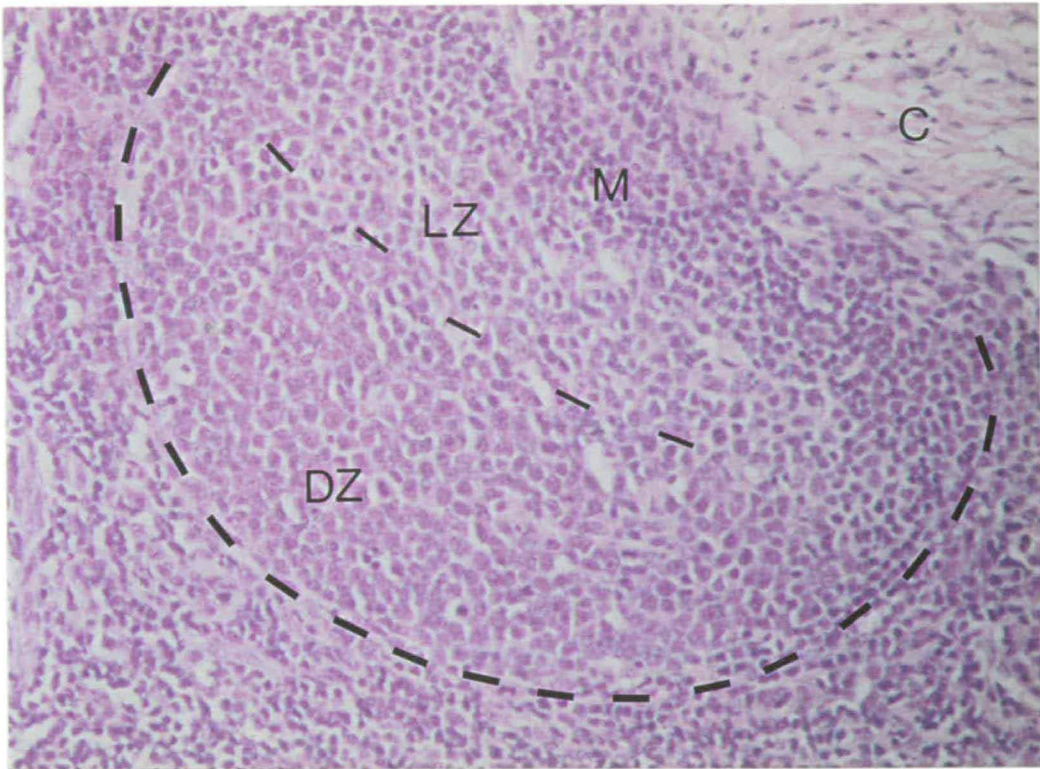


Fig. 3.8 Germinal centres with dark (DZ) and light zones (LZ) surrounded by a big mantle zone (M) in a draining node 16 days after immunisation. H+E, x125

zones surrounded by a small mantle zone (Fig. 3.7). The number of GCs was greater than the number of GCs seen on day 9. By day 16 (calf 12852), GCs with dark and light zones had become reactive with an increase in size and the appearance of a big mantle zone around them (Fig. 3.8). A large number of big (hyperplastic) GCs were observed in the day 16 node with some extending deep into the medulla, a feature not seen in the day 12 node. The GC of the deep cortex were less reactive than those of the superficial cortex. Some apoptotic cells occurred within the dark and light zones (Fig. 3.6).

#### **3.4.3.2 Responses in the paracortex**

The paracortex (T cell area) area was very small in the naive node (Fig. 3.9). It had increased by day 4 after immunisation and was hyperplastic by day 16 (Fig. 3.10). The paracortex was densely populated by small and big blasting cells by day 9 after immunisation. The high endothelial venules (HEV) which are the portal of entry for the circulation of lymphocytes to the node had increased in number by day 4 (Fig. 3.12) as compared to the naive node (Fig. 3.11). In the naive lymph node the HEVs were almost empty whereas they were full of lymphocytes in the day 9 node.

#### **3.4.3.3 Responses in the medulla**

There was a diffuse mixture of small lymphocytes and large non lymphocytic cells throughout the medulla in the naive node. The medullary sinuses were almost empty and sparsely populated by small lymphocytes on day 4 after immunisation (Fig. 3.13). The medullary sinuses were full of cells by day 9 (Fig. 3.14). There were few cells in the day 12 node whereas it was full of blasting cells by day 16 (Fig. 3.15).

The contralateral node responded similarly to the draining node by day 16 with the



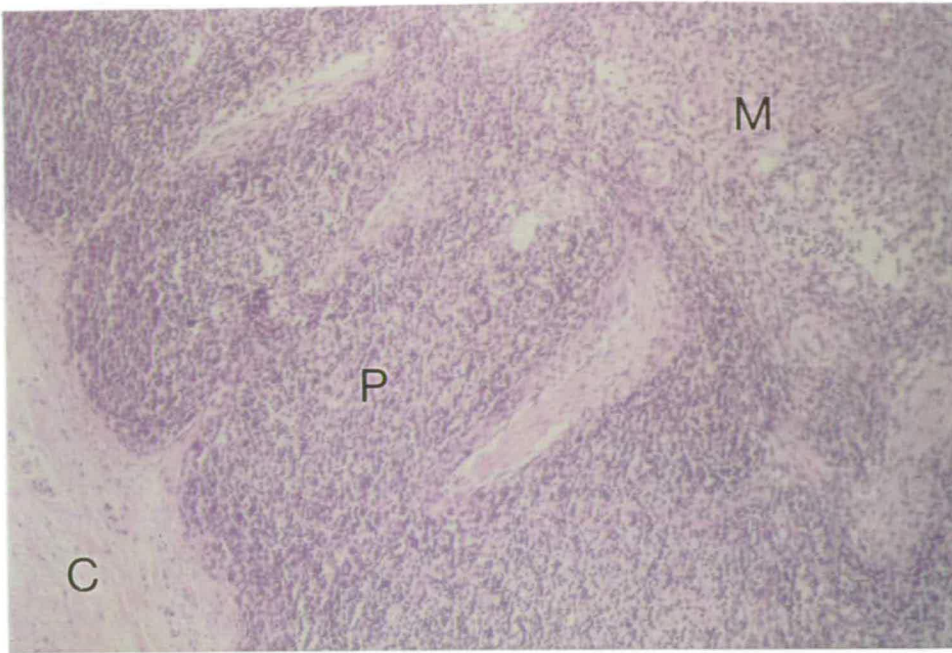


Fig. 3.9 Low power view of a normal lymph node. The paracortex (P) area is small. The capsule (C), paracortex and medulla (M) are visible within a single microscope field. H+E, x50

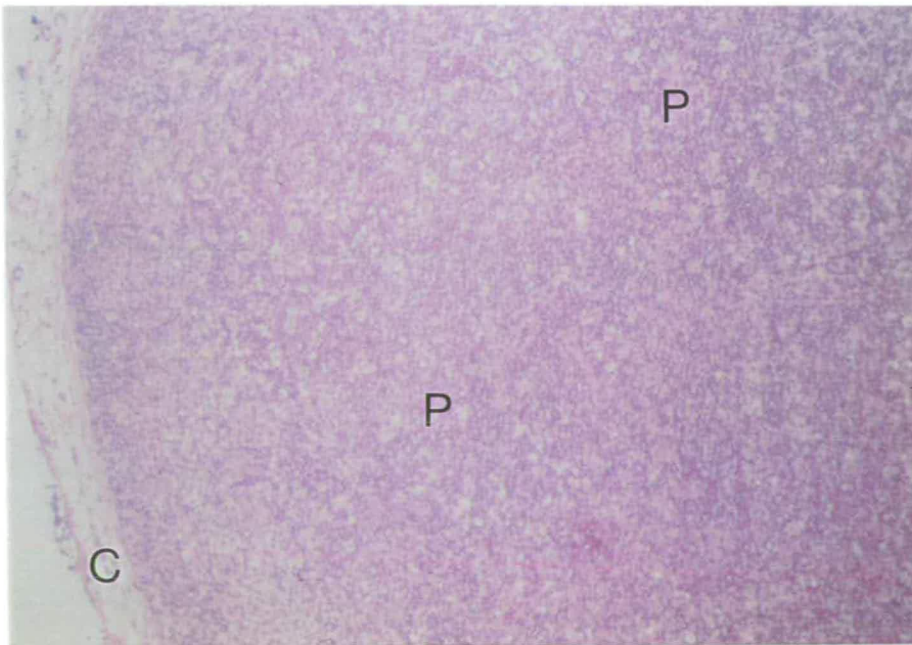


Fig. 3.10 Low power view of a draining lymph node day 16 after immunisation. Note the paracortex is large in comparison to a normal node (Fig. 3.9). The capsule (C) and paracortex are visible within a single microscope field. The medulla is not visible due to the increase in the paracortex. H+E, x50.

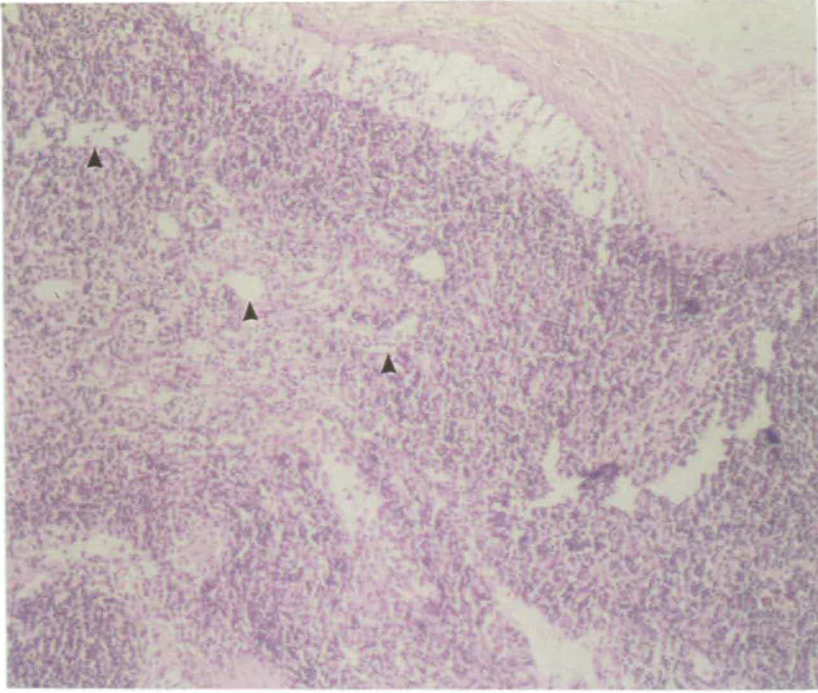


Fig. 3.11 Low power view of a normal node. Note high endothelial venules (↑). H+E, x50.

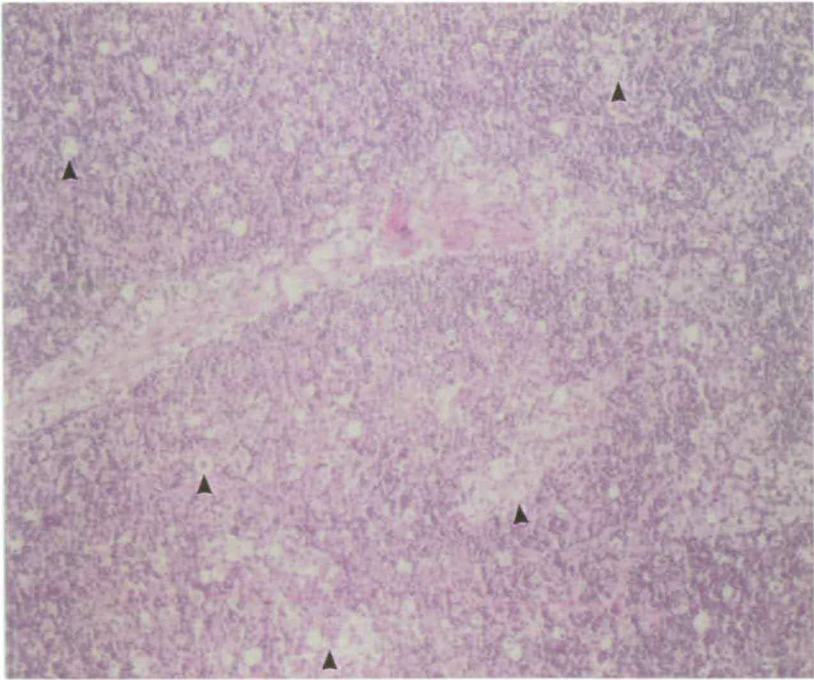


Fig. 3.12 Low power view of a draining node day 16 after immunisation. Note HEVs. H+E, x50



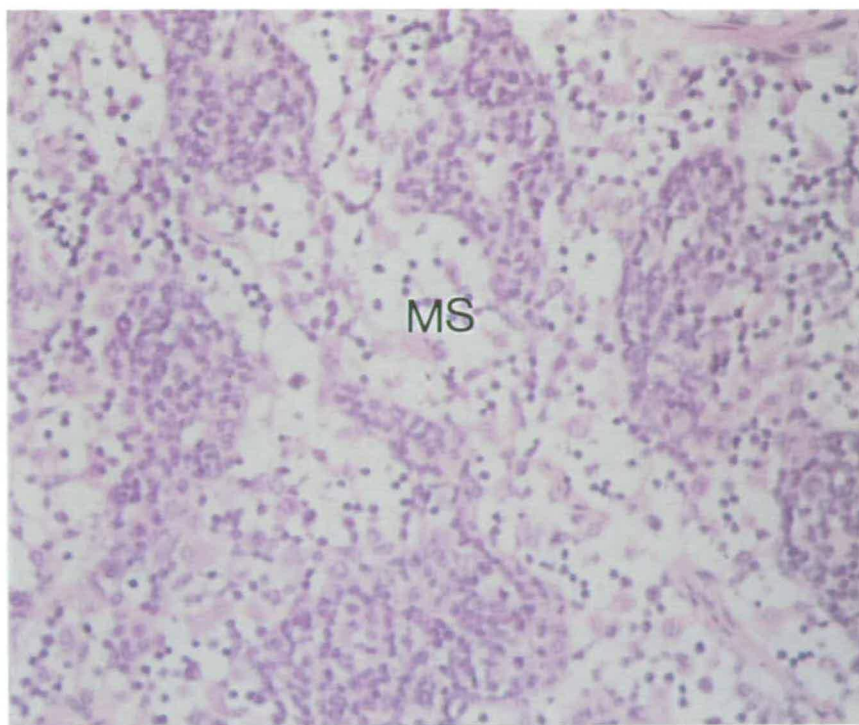


Fig. 3.13 Empty medullary sinuses (MS) in a draining lymph node day 4 after immunisation. H+E, x125.

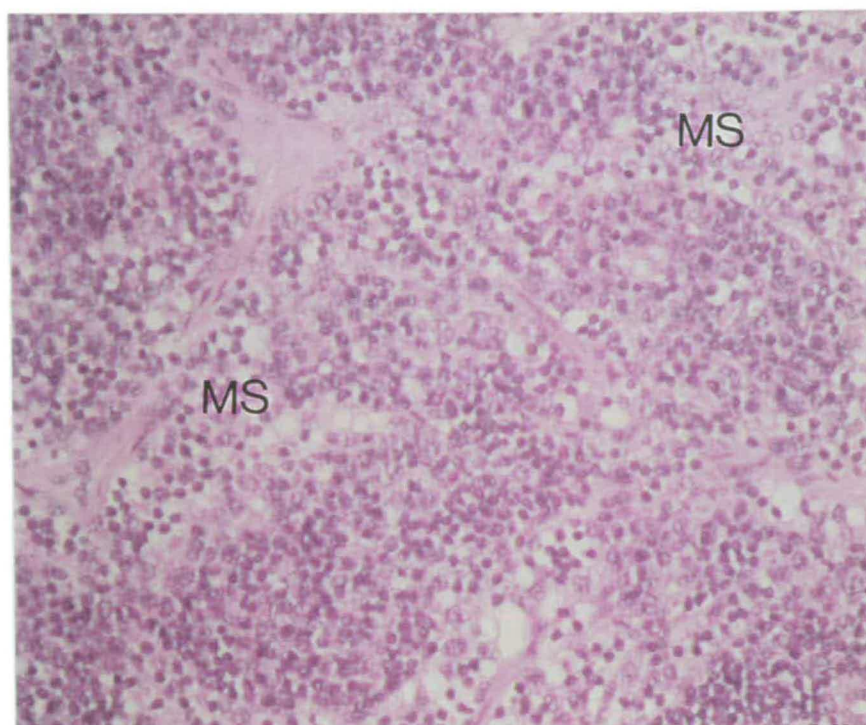


Fig. 3.14 Medullary sinuses full of cells in a draining lymph node day 9 after immunisation. Note lymphocytes are bigger than in Fig. 3.13. H+E, x125.



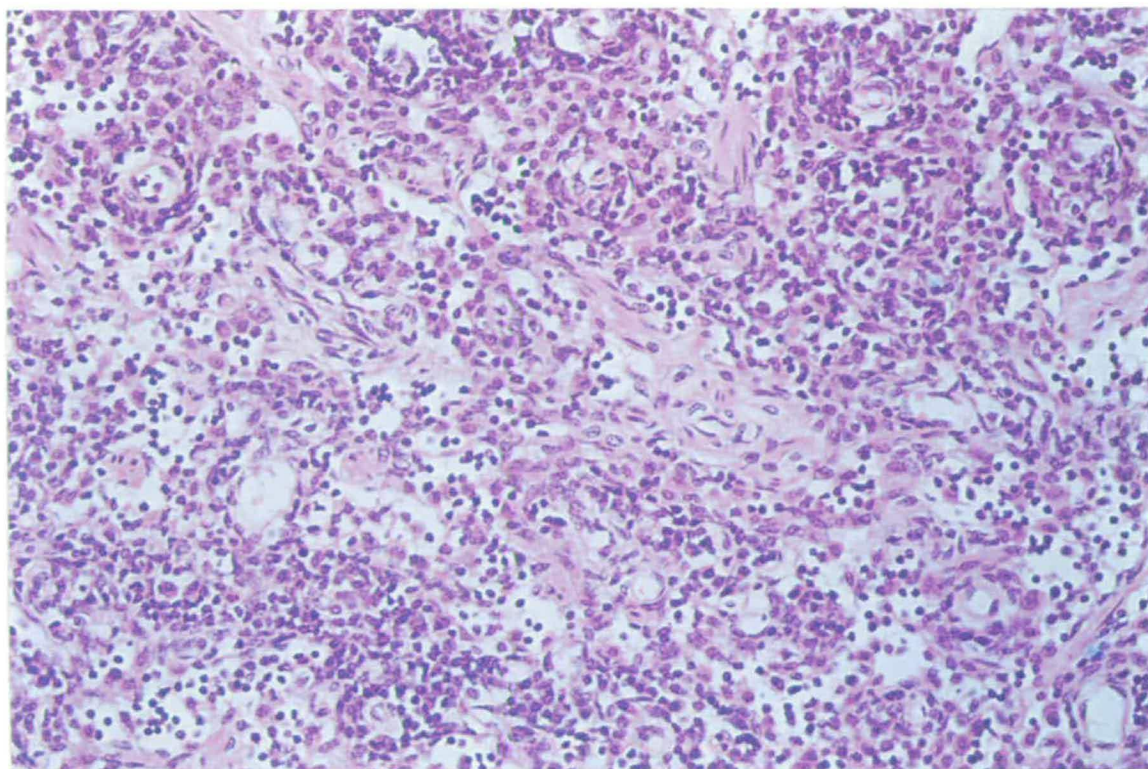


Fig 3.15 Medullary sinuses full of lymphocytes and macrophages in a draining lymph node day 16 after immunisation. H+E, x125.

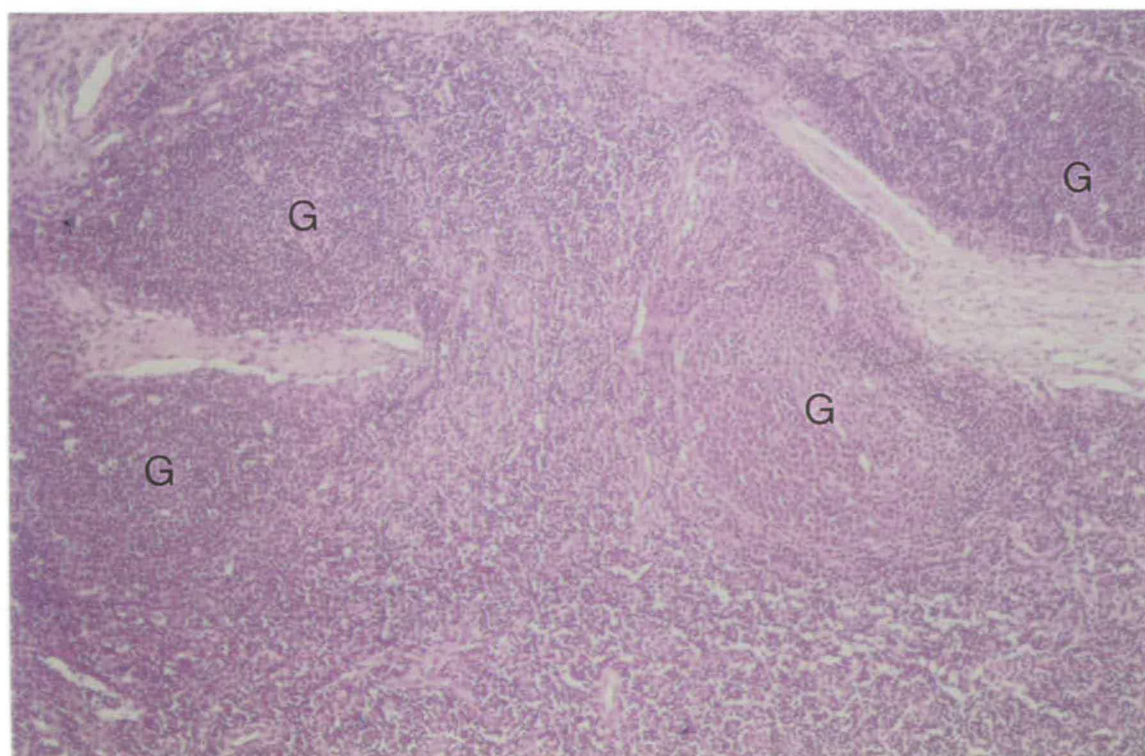


Fig. 3.16 Low power view of a draining lymph node day 16 after immunisation. Note 4 germinal centres in a single microscope field. H+E, x50.

exception that the hyperplasia of the GC was not as extensive as it was in the draining node (fig. 3.16).

#### **3.4.4 Immune responses in the lymph node draining the site of immunisation as examined by immunohistological techniques**

MAbs IL-A12 (CD4), CC63, SBUT-8 (CD8), IL-A24 and IL-A109 (macrophages and monocyte), VPM5 (Dendritic cells) and CC15 ( $\gamma\delta$  T cells) did not stain the paraffin sections even after the enzymatic digestion techniques. However, mAbs IL-A111, A-452, MIB-1, VPM30, IL-A15 and 1C7 were very efficient in identifying cells within paraffin sections of tissue.

##### **3.4.4.1 Responses in the primary follicle and germinal centres**

The constituents of a GC within a naive lymph node could be defined by several mAbs. MIB 1 consistently stained normal primary follicle/GC of all sizes. The cells stained by the MIB-1 (Ki-67, proliferating antigen) mAbs in the GC in humans have been defined as proliferating centroblasts within the dark zone area (Hardie *et al*, 1993).

A-452, a mAb for CD3 stained a few T cells in the primary follicles on day 4 (Calf 12848, Fig. 3.17), their number gradually increased by days 9 (Calf 12810, Fig. 3.18) and 12 (12851, Fig. 3.19) after immunisation. Many T cells occurred in the germinal centres by day 16 after immunisation (calf 12852, Fig. 3.20). Initially, T cells were present in the dark zone but later on more were observed in the light zone as the morphology of GC became more distinct (Fig. 3.20). CD3<sup>+</sup> T cells and CD3<sup>-</sup> B cells were observed in close proximity to each other.



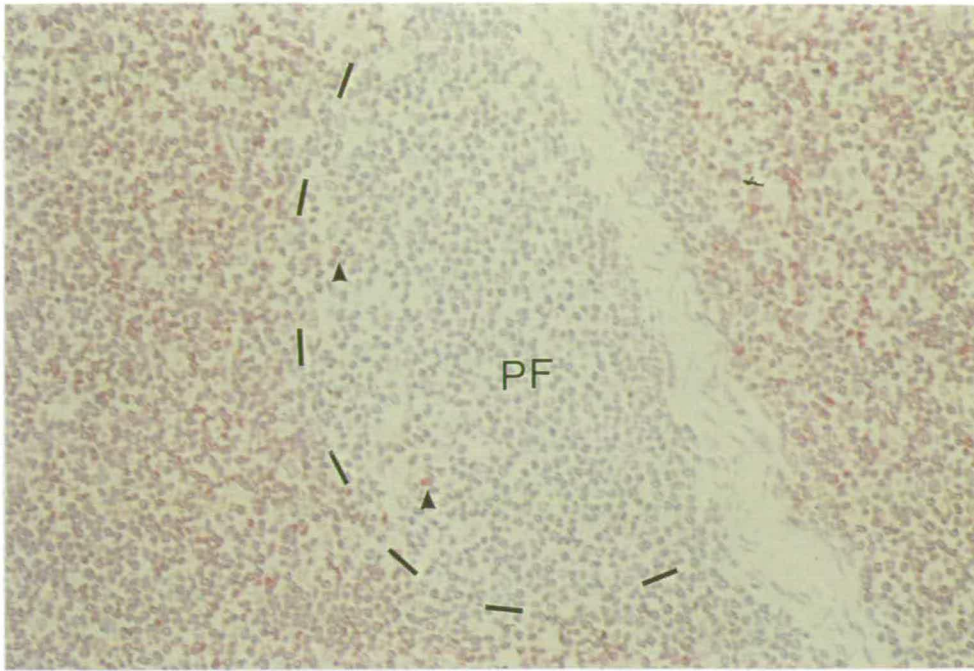


Fig. 3.17 T cells (red stain) in the paracortex and primary follicles (PF) by day 4 after immunisation. Note the number of T cells (↑) in the primary follicle. Vector red, x125.

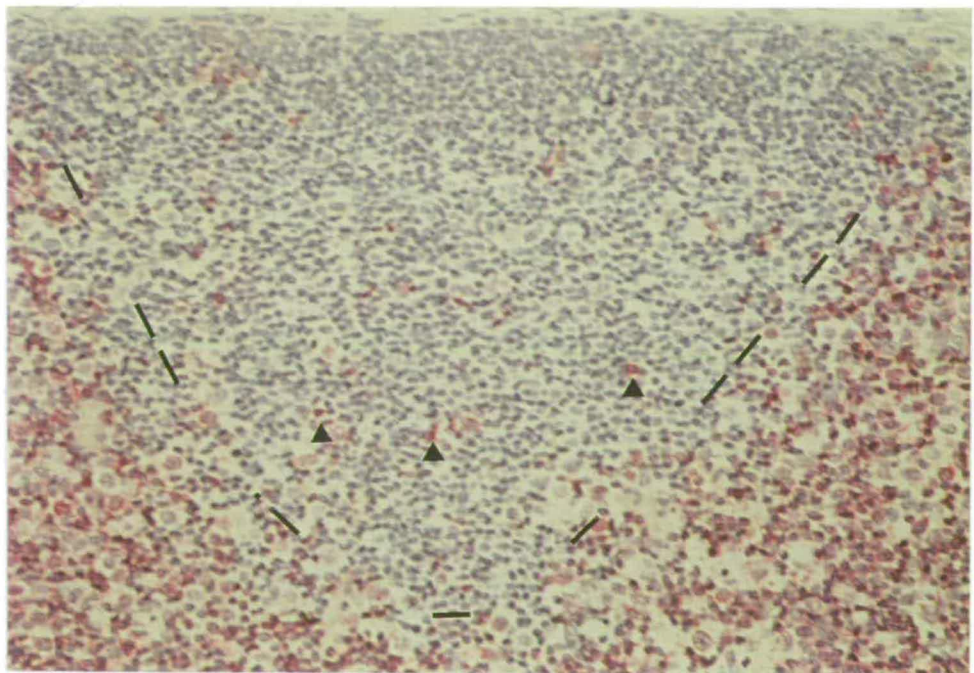


Fig. 3.18 T cells (red stain) in the primary follicle by day 9 after immunisation. Note the number of T cells in comparison to Fig. 3.17 in the follicles. Vector red, x125.

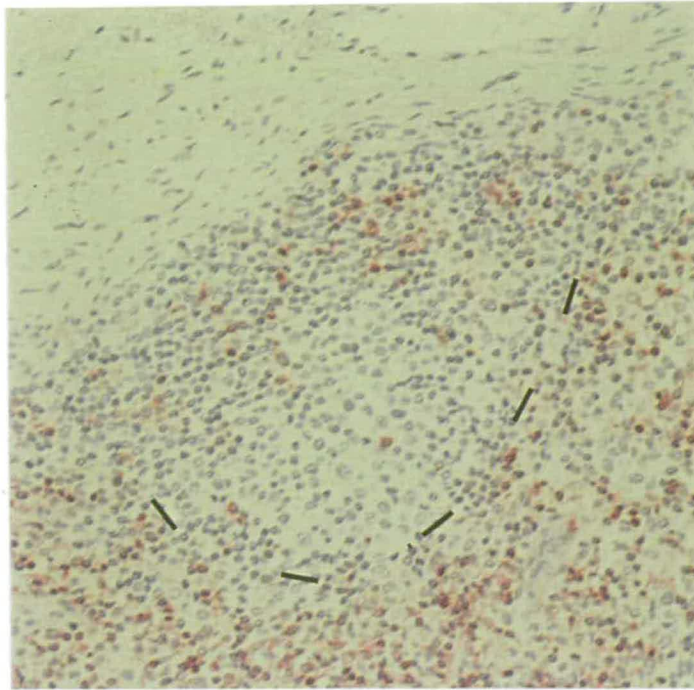


Fig. 3.19 T cells (red stain) in the germinal centre by day 12 after immunisation. Vector red, x125.

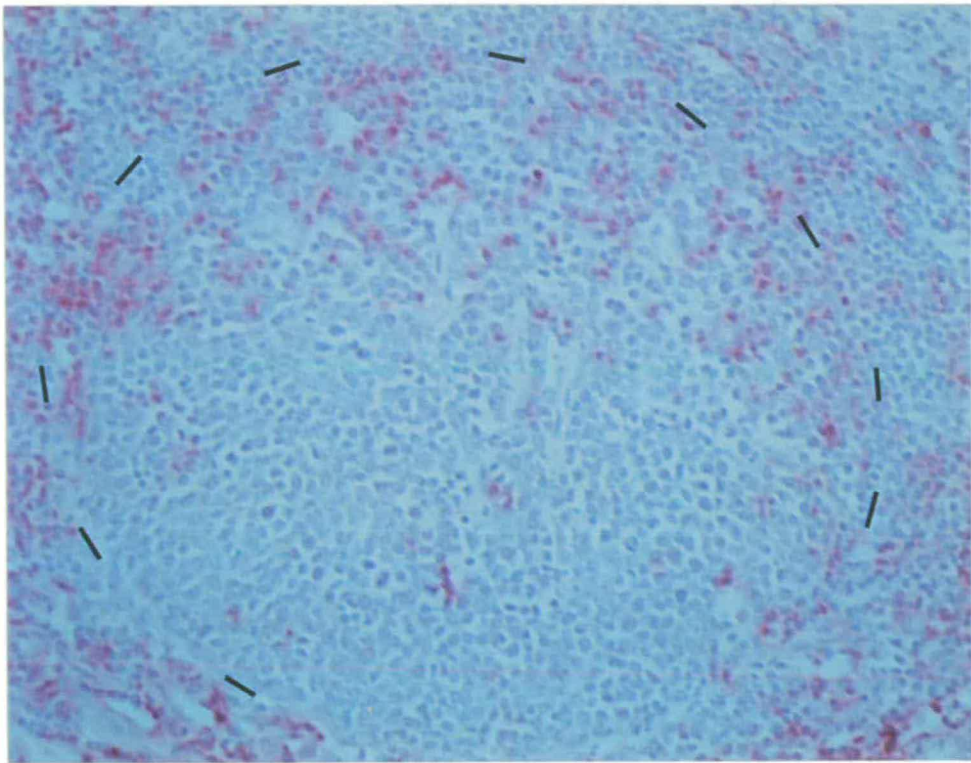


Fig. 3.20 T cells (red stain) in the germinal centre by day 16 after immunisation. Note the number of T cells in the light zone. Vector red, x125.

The number of MIB-1<sup>+</sup> cells was very low in the primary follicles by day 4 after immunisation (Fig. 3.21). They had increased by days 9 and day 12 after immunisation (Fig. 3.22) and a large number of proliferating cells were observed in the well developed germinal centres by day 16 after immunisation (Fig. 3.23). These MIB 1<sup>+</sup> cells were mainly observed in the dark zone with a few in the light zone of GC (Fig. 3.23). The number of proliferating cells increased in close association with the number of T cells in the follicles.

VPM30<sup>+</sup> B cells were observed in the basal light zone in some GC (Fig. 3.24) and in the light zone in most GCs (Fig. 3.25). Staining was weak in the basal light zone whereas it was strong in the light zone. The light zone pattern of staining was observed in most of the GCs. VPM30 recognises a differentiation Ag expressed on differentiated B cells mainly in the light zone of germinal centres. VPM30<sup>+</sup> B cells and CD3<sup>+</sup> T cells were observed in close proximity to each other in some sites (Fig. 3.26). VPM30 did not stain any of the cells in the PF/GC up to day 12 after immunisation and only stained GC cells on day 16 after immunisation.

The anti schizont mAb 1C7 stained a large number of cells in the germinal centre (Fig. 3.27). Tingible body macrophages stained by IL-A15 were present in the GC by day 9 after immunisation (Fig. 3.28). These bodies were mainly observed at the junction of dark and light zones; with a few in the light zone. There were few tingible bodies by day 16 in the GC.

#### **3.4.4.2 Responses in the paracortex and medulla**

The mAb IL-A111 (IL-2R) stained a few cells (1-2 per x10 field) in the naive lymph node. This pattern of staining changed by day 4 after immunisation with the



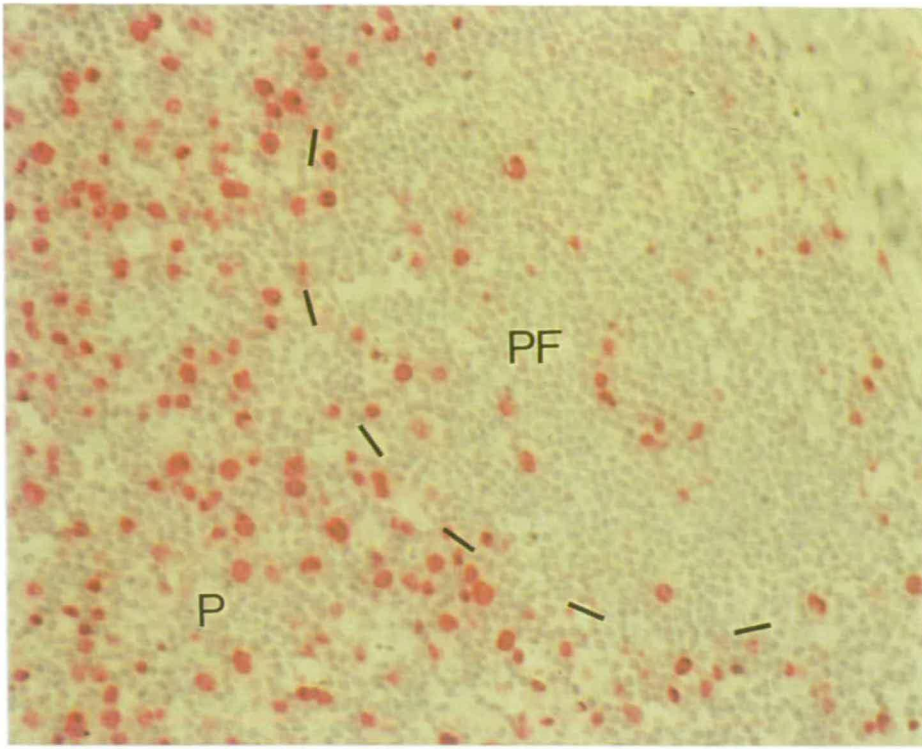


Fig. 3.21 MIB-1<sup>+</sup> proliferating cells (red stain) in the primary follicle (PF) and paracortex (P) by day 4 after immunisation. Note a few proliferating cells in primary follicle. Vector red, x125.

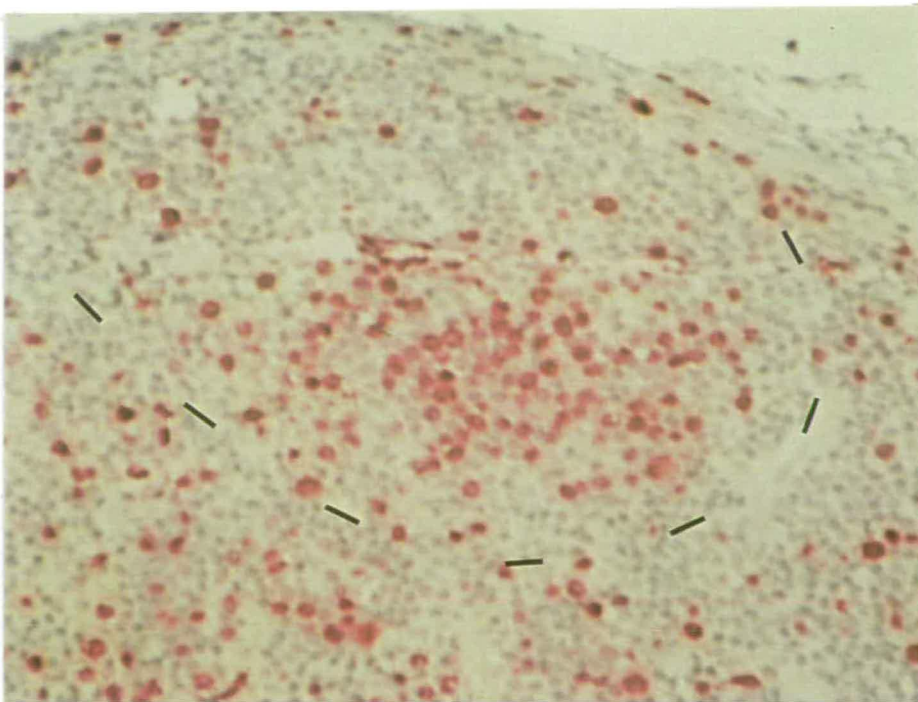


Fig. 3.22 MIB-1<sup>+</sup> proliferating cells (red stain) in the primary follicle and paracortex by day 12 after immunisation. Note a large number of proliferating cells in the primary follicle as compared to Fig. 3.21. Vector red, x125.

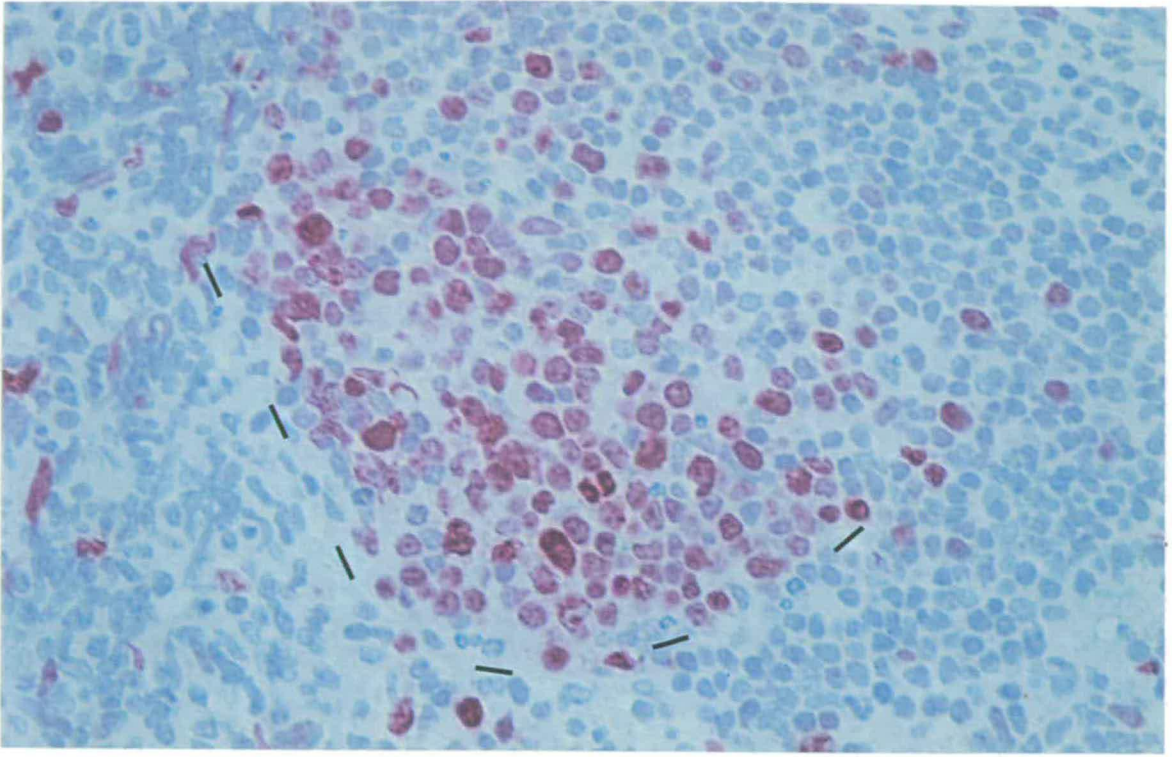


Fig. 3.23 Dark zone full of MIB-1<sup>+</sup> proliferating cells (red stain) by day 16 after immunisation. Note the number of proliferating cells in comparison to Fig. 3.21 and 3.22. Vector red.



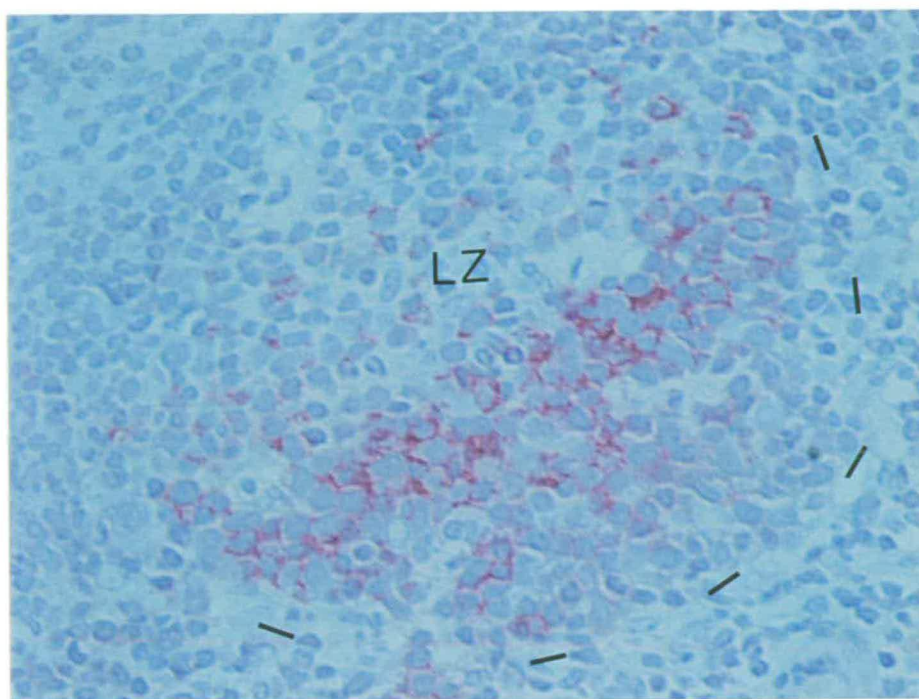


Fig. 3.24 VPM30<sup>+</sup> B cells (red stain) in the basal light zone of GC by day 16 after immunisation. Vector red, x125.



Fig. 3.25 VPM30<sup>+</sup> B cells (brown stain) in the light zone of GC by day 16 after immunisation. DAB, x125.

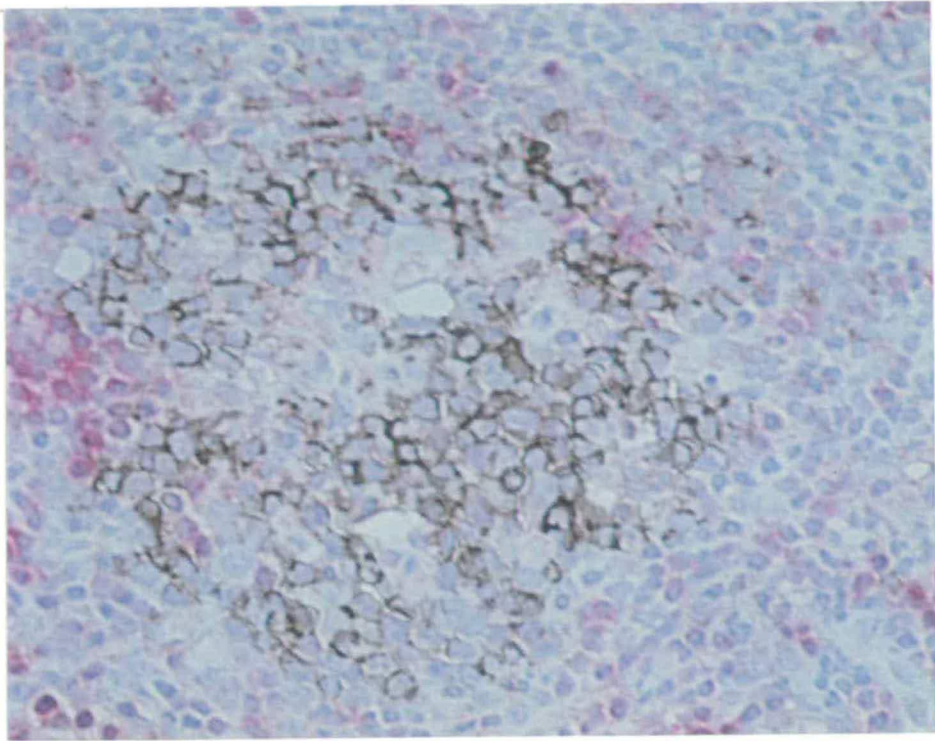


Fig. 3.26 Double stained DLN section by day 16 after immunisation. Note T cells (red stain) and VPM30<sup>+</sup> B cells (brown stain) lie close to each other at some places. Vector red and DAB, x250.



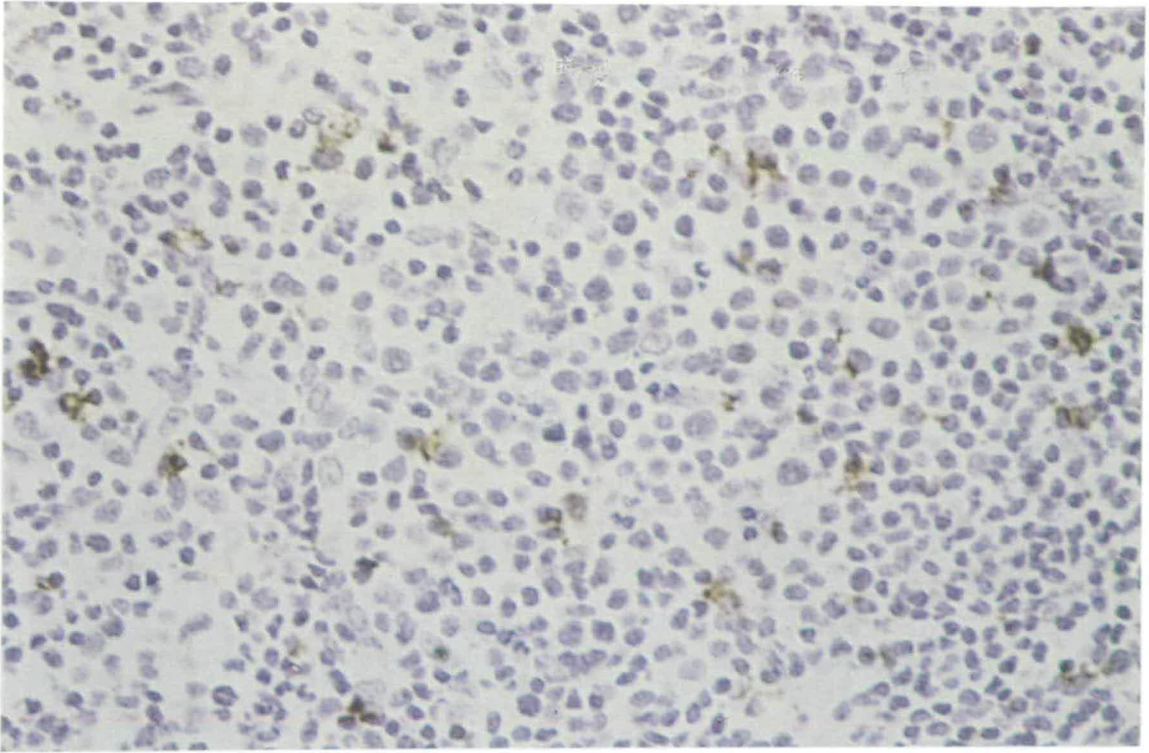


Fig. 3.27 1CT<sup>+</sup> cells (brown stain) within the GC of DLN by day 16 after immunisation. DAB, x250.

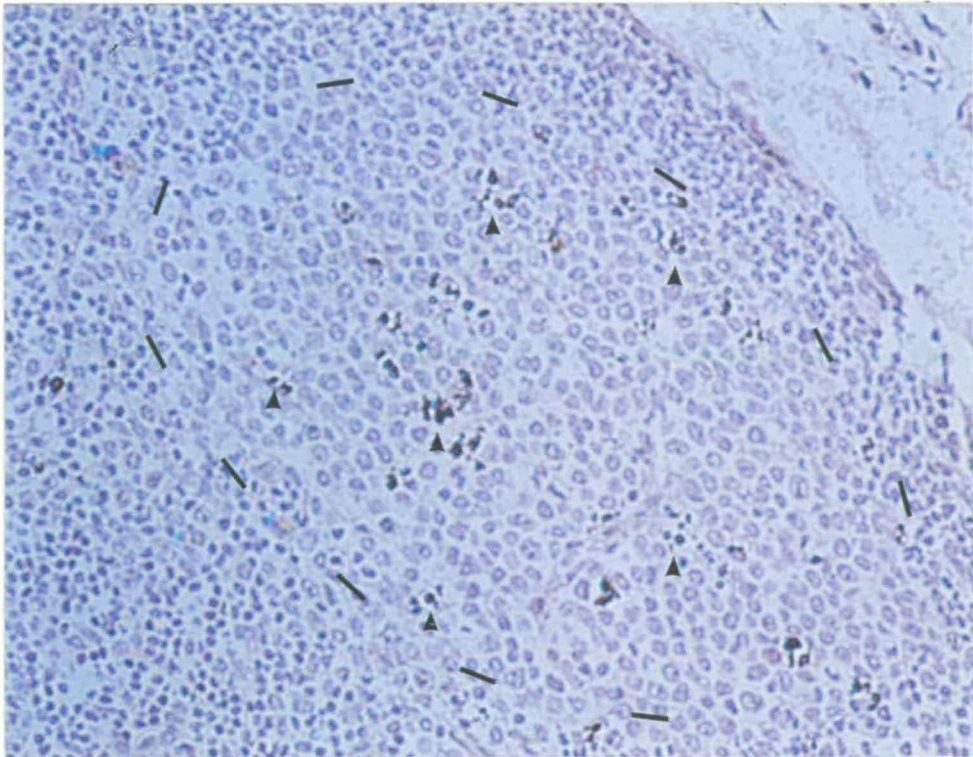


Fig. 3.28 Large number of tingible bodies (brown black stain) in the GC by day 9 after immunisation. DAB, x250.

appearance of IL-2R<sup>+</sup> cells in the paracortex specifically in and around HEVs (Fig. 3.29). These IL-2R<sup>+</sup> cells were CD3<sup>+</sup> (Fig. 3.29).

Staining with mAb MIB-1 revealed few proliferating cells in the medulla of lymph node on day 4 after immunisation but their numbers increased considerably by days 9 and 16. MIB-1 stained a large number of proliferating cells in the paracortex by day 4. Most of these proliferating cells were CD3<sup>+</sup> T cells as seen by staining with mAb A-452 on day 4 in the paracortex (Fig. 3.30) and by day 9 in paracortex (Fig. 3.31) and medulla (Fig. 3.32).

The T cells as stained by A-452 were densely populated in the paracortex by day 9 after immunisation as compared to naive. There were more CD3<sup>+</sup> T cells in the medulla by day 16 as compared to day 4.

1C7 stained schizonts in the medulla (Fig. 3.33) by day 16. These schizonts were in IL-A15<sup>+</sup> cells. IL-A15 recognises CD11b expressed on bovine monocyte/macrophages and granulocytes (Fig. 3.33). Some free schizonts were observed by day 9 and 12 along with some in the IL-A15<sup>+</sup> cells.

## **3.5 DISCUSSION**

### **3.5.1 Clinical observations**

All the four calves (12848, 12810, 12851 & 12852) showed mild parasitological reactions after immunisation as reported previously for cattle immunised with allogeneic *T. annulata* infected cell lines (Innes *et al*, 1989a; Brown, 1990; Nichani, 1995). The initial rise in temperature from 37.4°C on day 3 to 39.2°C by day 4 in calf 12848 and to above 40°C by day 10 in all the other three calves accompanied a



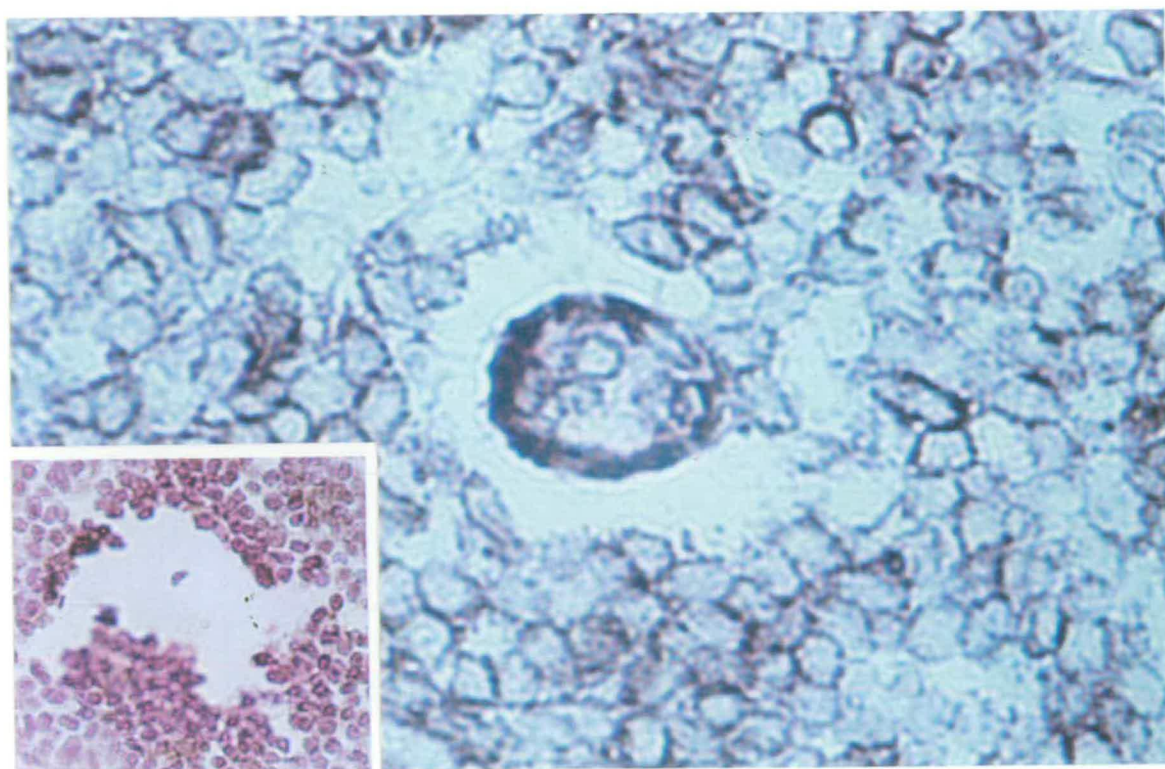


Fig. 3.29 Double stained CD3<sup>+</sup> (faint red stain) proliferating cells (black brown stain) in the HEV and the paracortex. Vector red and DAB, x400.

Inset- Double stained activated IL-2R<sup>+</sup> (brown stain) CD3<sup>+</sup> cells (red stain) in and around HEV. DAB and Vector red, x200.

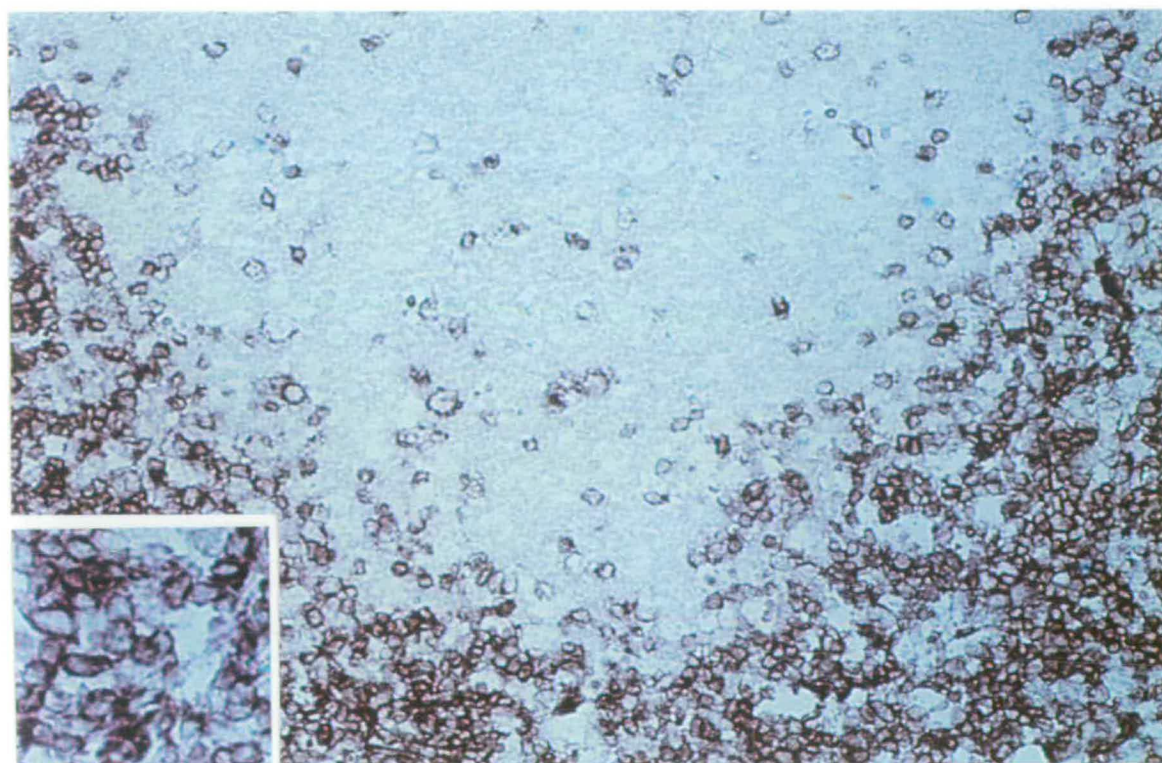


Fig. 3.30 Double stained proliferating CD3 (red brown) cells in the paracortex and few in the PF by day 4 after immunisation. x125.

Inset- High power view of proliferating (brown stain) CD3<sup>+</sup> cells (red stain) in the paracortex. Vector red and DAB, x250.



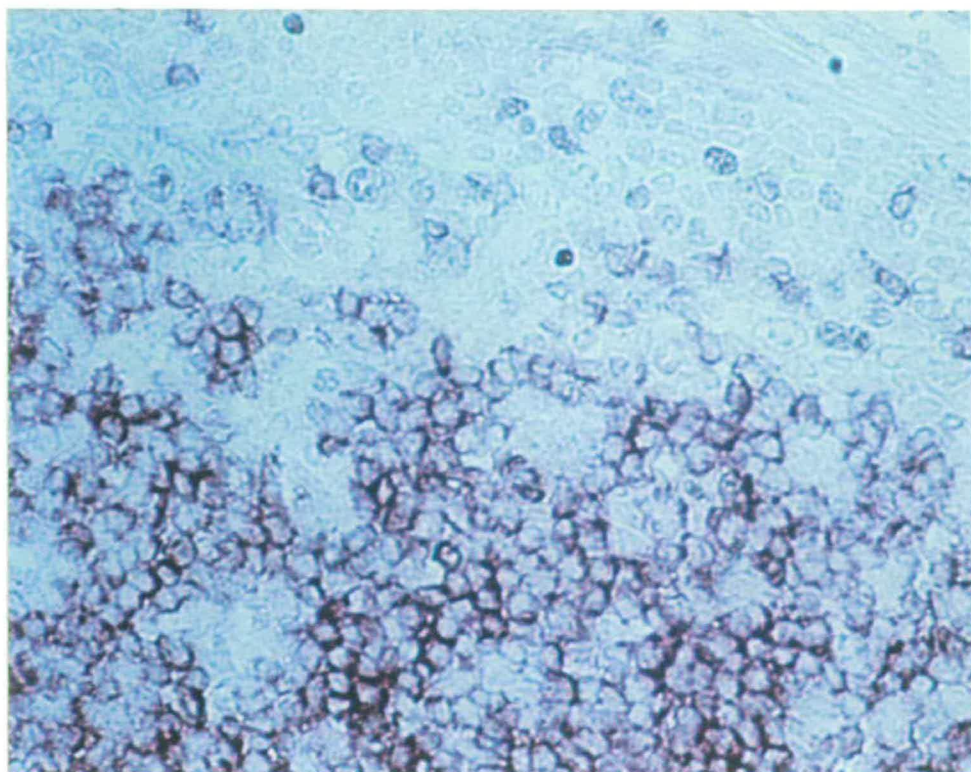


Fig. 3.31 Double stained proliferating (brown stain) CD3<sup>+</sup> cells (faint red stain) in the paracortex by day 9 after immunisation. x200.

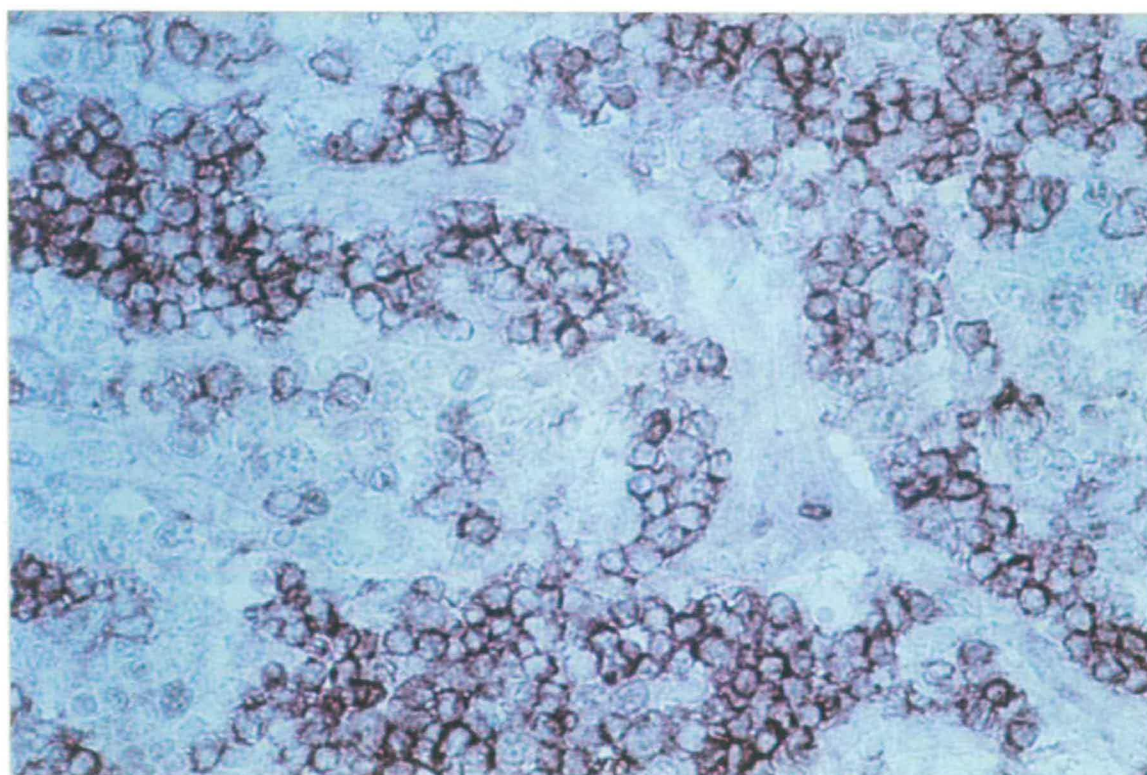


Fig. 3.32 Double stained proliferating (brown stain) CD3<sup>+</sup> cells (faint red stain) in the medulla by day 9 after immunisation. x200.

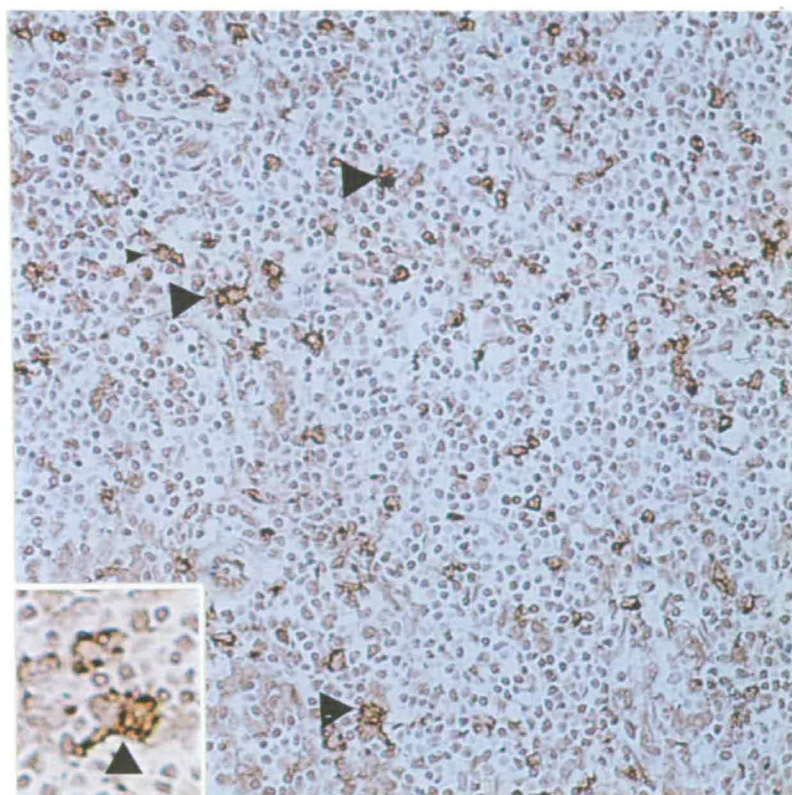


Fig. 3.33 Double stained 1C7<sup>+</sup> (red stain) and IL-A15<sup>+</sup> (brown stain) cells in the lymph node on day 16 after immunisation. Vector red and DAB, 125.  
Inset, high power view of the cells.

decline in TLC and TEC during the first 10 days could have been due to an allogeneic immune response to the MHC antigens of the immunising cell line in the DLN (Preston *et al*, 1983; Innes *et al*, 1989a). The absence of a temperature response in calf 12848 may have been due to the parasite being removed along with the lymph node on day 4. An increase in the number of lymphocytes in the medullary sinuses by day 4 and their peak by day 9 could have been due to anti MHC immune responses as were observed in the PBM and efferent lymph at this time after immunisation by Innes *et al*, (1989a) and Nichani (1995).

A second temperature peak in the 3rd week after immunisation accompanied by a drop in PCV and the appearance of the parasite in the peripheral blood has been reported as the second phase of the parasite specific immune responses (Preston *et al*, 1983; Innes *et al*, 1989a; Nichani, 1995).

### **3.5.2 Morphological responses observed with haematoxylin and eosin staining**

The most reactive changes were observed during the parasite specific phase of the immune response. There was hypertrophy of the paracortex, and hyperplasia and hypertrophy of GC with the presence of dark and light zones surrounded by a mantle zone. The GCs of the deep cortex were less reactive than those of the superficial cortex. Similar findings were observed by day 16 in trypanosomiasis (Moulten, 1986). The appearance of GC has been related to antigenic stimuli (Gadre *et al*, 1985) and its appearance within 16 days was reported to be because of primary immunisation in some other situations (Nieuwenhuis & Opstelten, 1984; MacLennan, 1990; Krose, 1987). The presence of GC within 6 days of infection with *T. parva* sporozoites has been attributed to tick antigen (Morrison *et al*, 1981b). In contrast to immunisation with cell lines of *T. annulata*, a total loss of cells with degenerative changes with the



involution of GC occurred after *T. annulata* sporozoite infection in the DLN (Eisler, 1988) and *T. parva* (Morrison *et al*, 1981b). Recently, in a more detailed study of DLN pathology, Campbell (1995) reported a decrease in the size and number of GC with no polarised hyperplastic GC by day 8 of a lethal *T. annulata* sporozoite infection. By day 10, not even a single normal GC could be found i.e. there was total destruction of the normal morphology of the GC. In contrast, the presence of the large number of reactive GCs observed in this study following immunisation with a *T. annulata* cell line clearly indicated the generation of immune responses in the DLN.

Degenerative changes in the GC by day 181 have also been observed in trypanosomiasis (Moulten, 1986) and during the late stages of HIV infection (Koopman *et al*, 1995).

A large number of cells in the medulla on days 4 and 9 after immunisation with cell lines may have been due to a cell shut down phenomenon in the DLN in response to the *T. annulata* infected cells. The same phenomenon was reported in the DLN after antigen challenge (Hall & Morris, 1965b; Hopkins *et al*, 1981) and after infection with sporozoites of *T. annulata* (Campbell, 1995; Nichani, 1995) and *T. parva* (Morrison *et al*, 1981a, 1981b). In contrast, Nichani (1995) did not observe any cell shut down in the efferent lymph after immunisation with *T. annulata* cell lines. The presence of a large number of cells in the medulla by day 9 could have been due to an increased supply of lymphocytes through the HEVs in response to inoculation of the cell line. This seems likely as the size of the node increased as compared to the normal lymph node and the node excised on day 4 after immunisation. Most of the HEVs were full of cells by day 9.

### 3.5.3 T cell responses in the DLN observed by immunohistology

In this study, activated T (IL-2R<sup>+</sup>) cells were observed initially at their normal anatomical sites in and around HEV and in the paracortex. Similar findings were first reported by Bogen *et al*, (1991), when they found IL-2<sup>+</sup> cells by day 4 after immunisation with protein antigen. In contrast to these findings, different patterns of localisation of IL-2R<sup>+</sup> cells were observed after lethal *T. annulata* sporozoite infection. These IL-2R<sup>+</sup> cells were present in the medulla and not in the paracortex by day 4 after infection (Campbell, 1995). Presence of IL-2R<sup>+</sup> cells accompanied the presence of the parasite in the medulla and the parasites were not observed in the T cell area. These IL-2R<sup>+</sup> cells were virtually absent by day 10 of infection and did not move to the B cell area at any time after inoculation of the sporozoites (Campbell, 1995).

In the present study, after the IL-2R<sup>+</sup> cells appeared in the paracortex, the number of T cells increased in the B cell area. The presence of T cells in the primary follicle of B cell area is believed to be critical for the formation of GCs as GCs do not form without the help of T cells (Jacobson *et al*, 1974). As antigen specific T cells and B cells are very rare *in vivo*, their physical linkage is unlikely to occur at random sites but in a specific area of lymphoid tissue. The GCs are the specialised area of the lymph node where T cell dependent B cell maturation and proliferation is believed to occur (Fliedner *et al*, 1964). Similar findings were seen in this study. T cells were rare in the PF of the normal node but their numbers gradually increased in the PF of immunised calves until day 16 after immunisation when at least 15-20 T cells were seen in each of the well developed GC. In general, T cells are believed to be antigen specific in the GC (Kathleen *et al*, 1993). B cells respond to an antigen early in the course of immunisation in the T cell rich area of the lymphoid organ (Liu *et al*, 1991) to obtain T cell help. However, T cells near the antigen responsive B cells in

the T cell area have not yet been studied for their antigen specificity. Even if they share antigen specificity in the T cell area, some functional properties like somatic mutation and maturation are unique to GCs. With the presence and increase of T cells in the GC, the number of Ki-67<sup>+</sup> proliferating cells increased leading to the formation of reactive GC after immunisation with *T. annulata* cell lines. Number of proliferating cells increased in response to antigen specific T cells in the GC (Fliedner *et al*, 1964). A large number of proliferating cells were present in the dark zone with an equal number of well proliferated B cells in the light zone of GC by day 16 after immunisation. Early proliferation of B cells is dependent upon expression of CD40 and the expression of CD40 ligand, which is mitogenic for B cells, on activated T cells (Armitage *et al*, 1993). An exactly similar situation may have occurred in this study when the B and T cells were in close proximity to each other in the dark as well as the light zones of the GC.

#### **3.5.4 Germinal centre reactions observed by immunohistology**

Identification of the functional compartments of a bovine GC was possible because of the availability of several mAbs which recognise bovine GC cells. By using mAb MIB-1, it was possible to compare bovine GC with human GC. MIB-1 stained dark zone of proliferating centroblasts in the human (Hardie *et al*, 1993) and bovine GCs (Campbell, 1995). The T cell zone was identified with a human polyclonal anti CD3 antibody (A-452, DAKO). The VPM30 mAb which recognises an antigen expressed on all peripheral blood and efferent lymph B cells (Naessens & Howard 1991; Nichani, 1995) stained cells only in the light zone in most of the well developed GCs (Campbell, 1995). It also stained basal light zone in some of the GC in this study.

VPM30 recognised early activated T cells in PBM upon Con A stimulation (Campbell

*et al*, in press) but it did not stain activated T cells in the GC in the present study. It is thought that the expression of antigen recognised by the VPM30 on the B cells is likely to be dependent upon T cell help in the GC (Campbell, 1995). The molecule VPM30 was expressed on B cells in the light zone (Campbell, 1995); this expression is thought to be cytokine dependent (Hodgkins *et al*, 1991). An exactly similar situation was observed in this study after immunisation with cell line where VPM 30 stained B cells in close association with CD3<sup>+</sup> T cells only in the light zone and only on day 16. The possibility that this process is cytokine dependent will be discussed in chapter 5.

To summarise, the DLN responded to inoculation of the *T. annulata* infected cell line with the presence of IL-2R<sup>+</sup> and proliferating CD3<sup>+</sup> cells in the paracortex by day 4 and the appearance of reactive GCs by day 16 after immunisation. Assessment of the functional activation state of these DLN cells was only possible by undertaking the *in vitro* functional assays which are described in the next chapter together with analysis of the cell cycle of proliferating cells.

## **CHAPTER 4**

### **FUNCTIONAL ACTIVITY OF DRAINING LYMPH NODE CELLS FOLLOWING IMMUNISATION WITH *THEILERIA ANNULATA* INFECTED CELL LINES**

## 4.1 INTRODUCTION

It has been well documented in the literature that CTL mediated immune responses occur in the PBM (Preston *et al*, 1983; Innes *et al*, 1989a) and in the efferent lymph (Nichani, 1995) after immunisation with *T. annulata* infected cell lines. The cells leaving the DLN in the efferent lymph have been shown to be specific initially for the MHC antigens of the immunising cell line and later to the autologous parasitised cells (Nichani, 1995). Histological evidence for T cell activation after immunisation with cell lines has been shown in chapter 3. Thus a large number of MIB-1<sup>+</sup> cells, T cells and IL-2R<sup>+</sup> cells were found in the paracortex by immunohistology on and after day 4 following immunisation. Different levels of serum antibody have been reported after immunisation with different cell doses at different passages of a *T. annulata* infected cell line (Shukla *et al*, 1989).

Since the state of functional activation of DLN cells or their specificity to antigen could not be defined by immunohistology, the following *in vitro* assays were performed to explore their state of activation and to confirm the findings described in chapter 3.

## 4.2 EXPERIMENTAL DESIGN

The following assays were undertaken to define the activation state of DLN cells: *In vitro* assessment of the proliferation of DLN cells in response to recall antigens; flow cytometric analysis of the cell cycle of DLN cells and the number of apoptotic cells (as measured by DNA histograms). Antibody titers in the serum were assessed by the immunoperoxidase test.

## **4.3 MATERIALS AND METHODS**

### **4.3.1 Animals and immunisation**

All the animals were the same as used in the chapter 3.

### **4.3.2 Isolation of DLN cells for proliferation assays**

Freshly excised pieces of lymph nodes from calves 13046, 13054, 12848, 12810, 12851 & 12852 were cut into approximately 1cm<sup>2</sup> blocks and placed in a sterile 20ml tube containing 10ml PBS supplemented with 1% antibiotics (10,000 IU/ml penicillin, 10mg/ml streptomycin). These nodes were the same as those used for immunohistology. The nodes were washed twice in PBS to remove the surface blood and then placed in a sterile petri dish containing 10 ml PBS with antibiotics. The tissue was then chopped with sterile scalpels, first removing the capsule wall. All the minced tissue was further homogenised into an autoclaved tissue homogeniser (Jencons Scientific). The cells were washed 3 times in PBS before proliferation assays described in this chapter or cryopreservation in DMSO for future mRNA analysis (chapter 5).

#### **4.3.2.1 *In vitro* proliferation assay**

Assays were performed essentially as described by Glass and Spooner (1990). Briefly, the DLN cells (1x10<sup>6</sup>/ml, 100µl/well) were used as responders and stimulated by the following (100µl/well) in quadruplicate wells : 1) Con A at 4µg/ml; 2) recombinant IL-2 (Boehringer Manhein) at 40 IU/ml; 3) irradiated autologous *T. annulata* infected cells (1x10<sup>5</sup>/ml, 100µl/well); 4) irradiated allogeneic *T. annulata* infected cells belonging to the line used for immunisation (1x10<sup>5</sup>/ml, 100µl/well). The *T. annulata* infected cells were irradiated at 7500 rads. Responder cells (DLN cells) were incubated alone as a negative control. Tests were performed in 96-well flat bottom

plates (Inter med, Nunc) in a final volume of 200µl/well. 100µl TC medium was added to each well containing either responder or stimulators alone. The plates were incubated for 72 hrs at 37<sup>0</sup>C in a CO<sub>2</sub> incubator. The cells were labelled with methyl <sup>3</sup>H thymidine (Amersham International, Amersham) for 6 hrs and harvested onto a glass filter paper (Wallac) using a cell harvester (Inotech AG). The filters were dried, sealed in the plastic bags with 3ml of scintillant (Optiphase safe, LKB Scintillation products) and counted using a liquid scintillation counter (Microbeta, Wallac).

**4.3.3 Flow cytometric analysis of the cell cycle and apoptotic cells**

Cell kinetics and apoptosis in DLN cells was measured by the binding of DNA specific fluorescent dyes to nucleic acids using flow cytometry as described by Hedley (1983, 1989). The amount of dye bound was proportional to the amount of DNA, the number of cells in a particular phase of cell cycle or apoptosis could be identified by the relative fluorescence.

**4.3.3.1 Isolation and staining of nuclei**

Nuclei were obtained from cells prepared from sections (50µm) cut from the same paraffin blocks as used for histological examinations. The sections were floated on to the glass slides and dried for 1hr at 80<sup>0</sup>C. Section were rehydrated as following:

Xylene	2 times	10 minutes each
100% alcohol	1	10 minutes
99%alcohol	1	10 minutes
95% alcohol	1	10 minutes
Distilled water	2	10 minutes

The rehydrated sections were then transferred using a clean pasteur pipette into a 4 ml tubes (Falcon) containing pepsin digestion solution. These tubes were incubated



for 1 hr at 37°C, vortexing after every 10 minutes. The cells were washed twice with 1 ml PBS by centrifuging at 100g for 10 minutes and finally resuspending in 1 ml PBS. The nuclei suspension was filtered through cotton wool as follows. A piece of clean cotton wool was placed in the barrel of a 10ml syringe and the plunger replaced in the barrel, flattening the cotton wool. The plunger was removed and the cell suspension pressed through the syringe. A separate filter was prepared for each sample.

Nuclei were stained in 100µg propidium iodide (Sigma) in 1ml PBS containing RNAase. The tubes were wrapped in aluminium foil and incubated for 1 hr at room temperature. Nuclei were centrifuged to remove stain and resuspended in PBS. Samples were run immediately.

#### **4.3.3.2 *Flow cytometric analysis***

Flow cytometric analysis of DNA was carried out using a Flow cytometer (FACScan, Becton Dickinson). This flow cytometer was used for DNA analysis with the laser at 488nm. PI fluorescence was detected at 570nm (FL2 detector). PI stained nuclei produced a typical DNA histogram, with fluorescence area (intensity) on the x-axis and total number of nuclei on the y-axis. The first peak on the x-axis corresponds to diploid nuclei in the G<sub>0</sub> or G<sub>1</sub> phase of cell cycle and a small peak towards the end of x-axis corresponds to nuclei in G<sub>2</sub>/M phase of cycle. The S-phase lies in-between the two peaks (Fig. 4.1 ).

##### **4.3.3.2.1 *Analysis of the cell cycle***

DNA fluorescence was measured on a linear rather than a log scale. The problem of dissecting the DNA histogram into its component parts consists of separating the

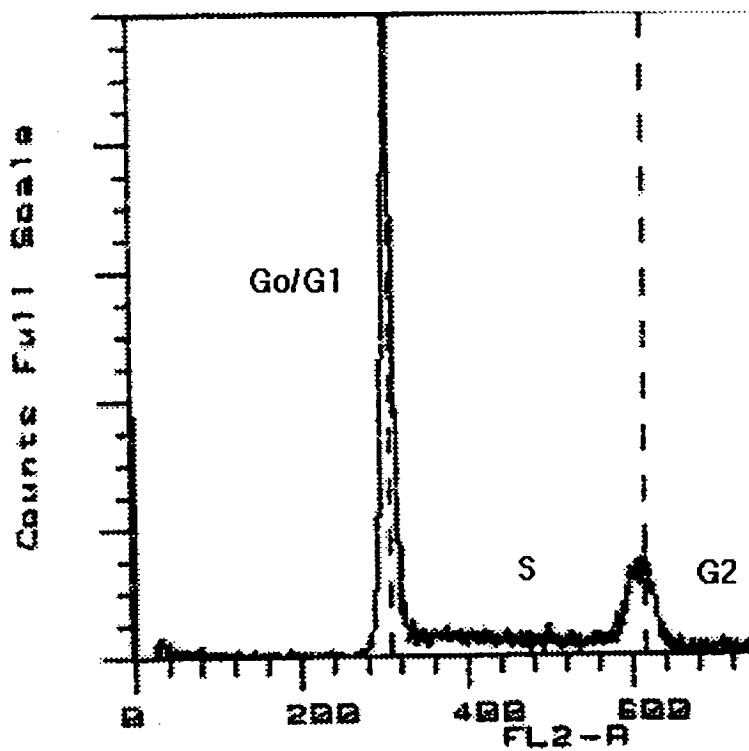


Fig. 4.1 Sample DNA histogram, with  $G_0/G_1$ , S, and  $G_2$  peaks marked. Dotted lines represent computer determined median  $G_0/G_1$  and  $G_2$  values.

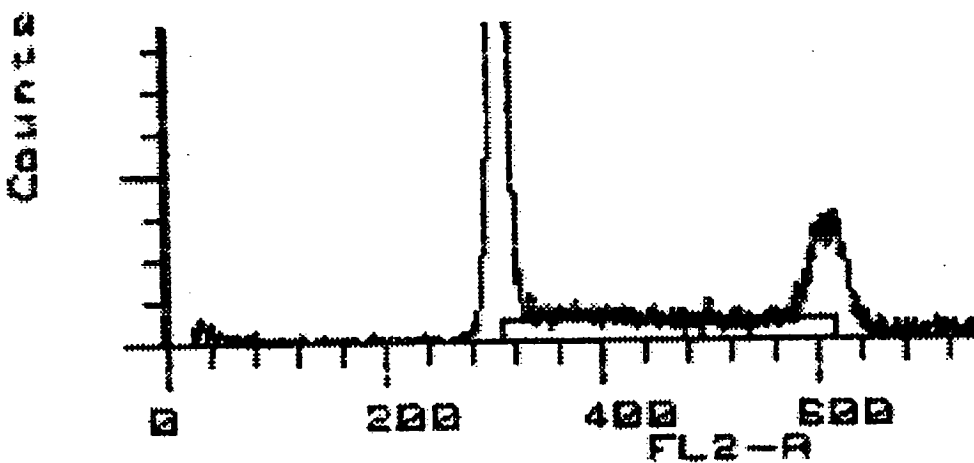
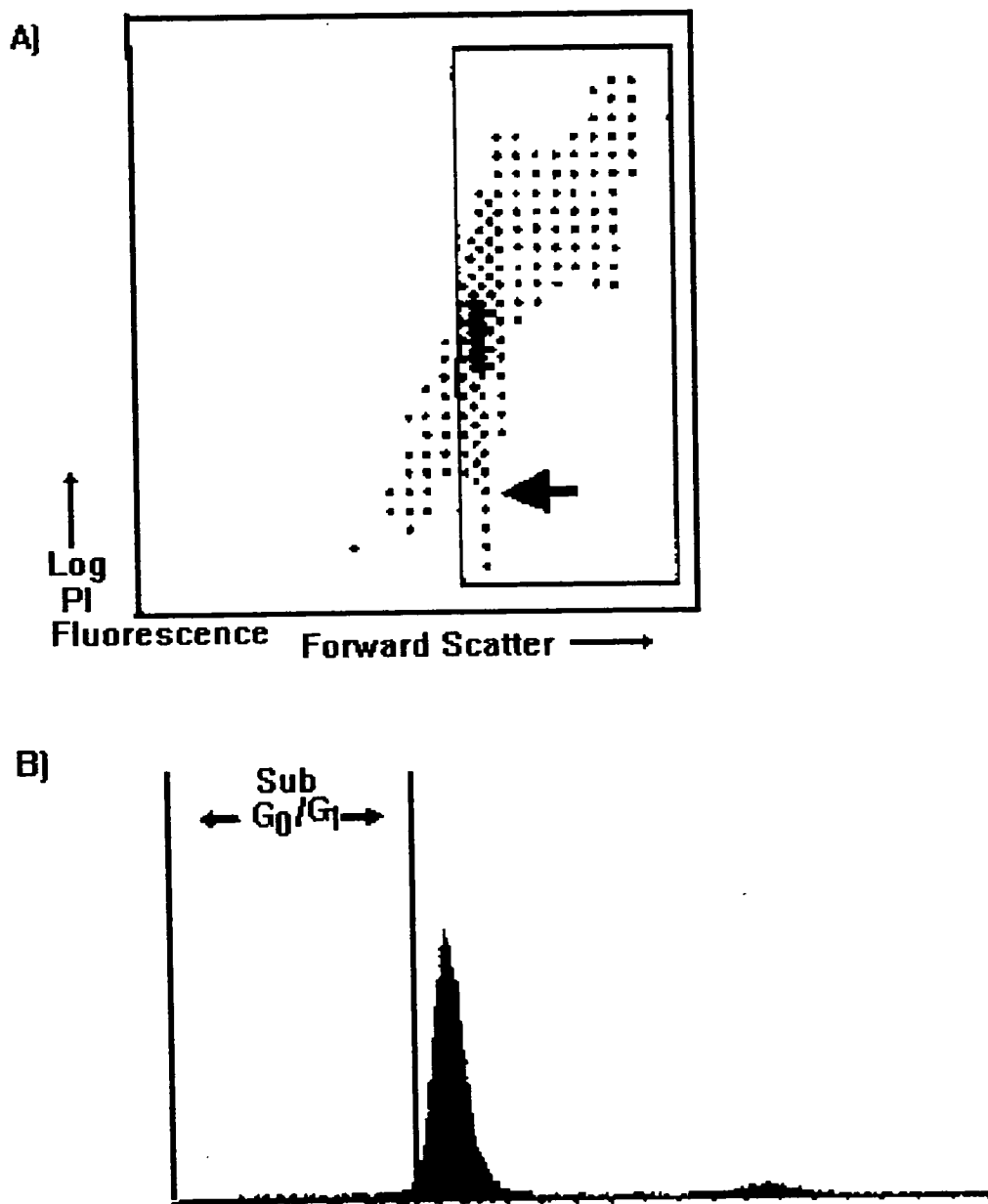


Fig. 4.2 Identical sample to Fig. 4.1 enlarged to show rectangle fitted under S-phase

DNA content of cells in early S phase from those in  $G_0/G_1$  phase and those in late S phase from those in  $G_2/M$  phase. There are a number of methods available for determining the areas of the various peaks. Most of these rely on mathematical models and have a degree of error, still a useful comparison can be drawn from identically processed samples analysed with the same model. In this study, the number of nuclei of the DNA histograms were determined using the method described by Baisch *et al*, (1975). It is usually assumed that the cells in the  $G_1$  and  $G_2$  phases are distributed normally and in practice this proves to be a reasonable assumption. Essentially the method of Baisch (1975) identified the  $G_1$  and  $G_2$  peaks as normal distributions and fitted a rectangle between the two peaks to estimate the S phase peak. Practically, the model was fitted to the histogram by the computer (Fig. 4.2).

#### **4.3.3.2.2 Analysis of apoptosis**

Apoptotic nuclei appear as a sub  $G_0/G_1$  or hypodiploid peak on a DNA histogram due to the condensation of nuclear material in the cells. Nuclei were gated for apoptosis using a log scale for PI and a linear scale for FSC. Apoptotic nuclei were of similar size and therefore appeared under the main population of cells of forward scatter on the x-axis and fluorescence on the y-axis (Fig. 4.3a). Debris appeared on the left of the main population because of its small size. The sectioning might have led to a higher yield of sheared nuclei, still it was useful as all the samples were treated identically. The measurement of the apoptotic peak (Sub  $G_0/G_1$ ) was carried out using the histogram obtained in the cell cycle analysis (Fig. 4.3b). The hypodiploid region was chosen on the normal nodes and remained unchanged throughout all the samples. Apoptosis was assessed in all the 5 sections (nodes) on 2 different occasions.



**Fig. 4.3**

**A:** Schematic representation of nuclei gating for apoptosis analysis. Apoptotic nuclei (←) have same size as normal nuclei but are less fluorescent. Smaller debris is excluded.

**B:** DNA histogram from normal lymph node showing sub  $G_0/G_1$  areas corresponding to hypodiploid nuclei as shown in A.

#### **4.3.4 Immunoperoxidase test for antibodies**

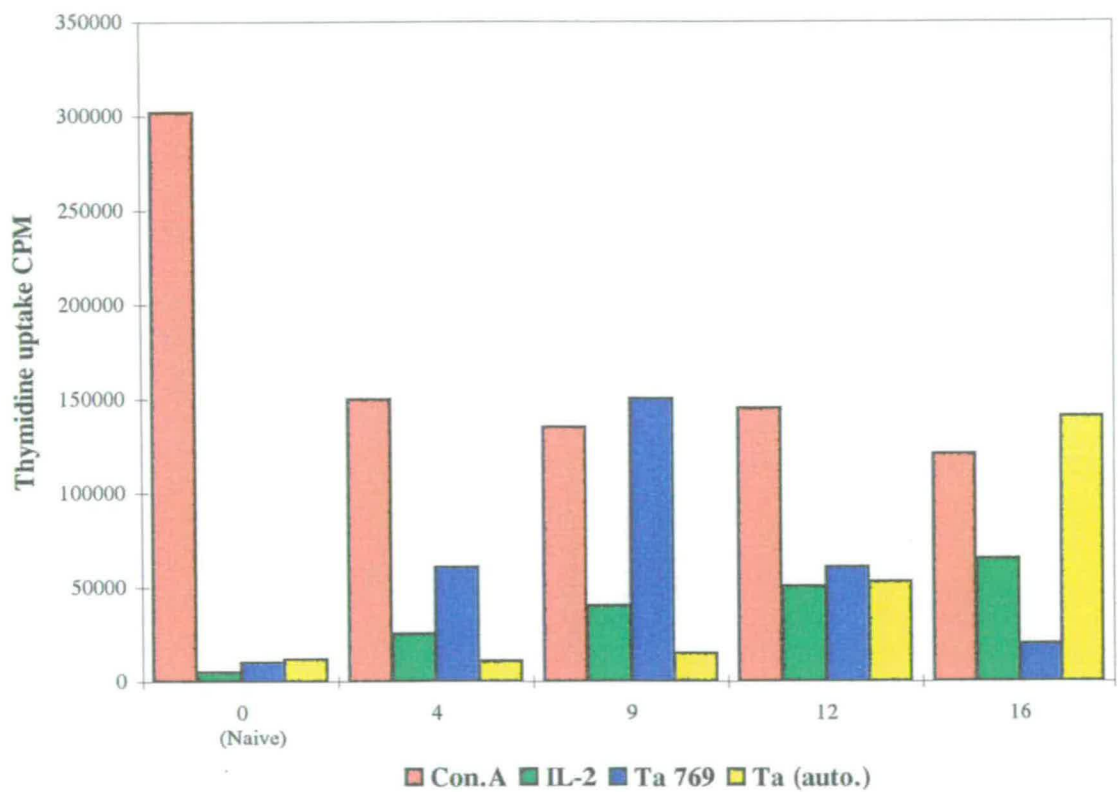
Antibody titers from the serum of immunised animals were assayed using a immunoperoxidase test (Campbell *et al*, 1994b). Antigen coated slides were prepared as follows (Burridge and Kimber, 1972). *T. annulata* (Gharb) infected cells were washed twice in sterile PBS and once in PBS supplemented with 1% BPA. The cell pellet was resuspended in three times its volume of PBS/BPA. Multiwell slides (Flow Laboratories) were coated with this cell suspension by applying 20 µl to each well and then immediately removing it. The slides were dried quickly and fixed in acetone (BDH) for 15 min., air dried and wrapped in medical tissue wipes. The slides were then kept in a polythene bag containing silica gel and stored at -20°C until used.

Antigen slides were removed from -20°C and held at 4°C for 30 minutes and then at room temperature for a further 30 min. The slides were rinsed in PBS. A four fold dilution of serum to be tested was prepared in PBS. Antigen slides were placed in a humid chamber and incubated with 20 µl of normal mouse serum (1:10 in TBS) for 10 min. and then incubated with the diluted serum to be tested for 30 min. This was then followed by the method described for immunostaining in chapter 3 (ABC-HRP) with the exception of secondary antibody, mouse antiovine IgG (Sigma: B-9780). The highest dilution of the test serum which gave positive staining was recorded.

### **4.4 RESULTS**

#### **4.4.1 Proliferation assay**

The proliferative ability of the DLN cells was assessed in the *in vitro* assays in response to Con A, rIL-2, *T. annulata* (H) immunising cell line (Ta 769) and *T. annulata* autologous cell line (auto.)(Fig. 4.4). The DLN cells of the naive animal proliferated very well in response to Con A but negligibly to IL-2, *T. annulata* (H)



**Fig. 4.4** Proliferative responses of DLN cells (days 0,4,9,12 and 16) to Con.A, *T.annulata*immunising (Ta 769) and *T. annulata*autologous (auto.) cell lines.

769 and *T. annulata* (auto.) cell lines. DLN cells (calf 12848) harvested 4 days after immunisation showed a reduced response to Con. A, slight proliferation to IL-2 and a strong proliferation to *T. annulata* (H) 769. DLN cells (calf 12810) harvested 9 days after immunisation showed peak proliferation against the immunising cell line with an increased response to IL-2. DLN cells harvested 16 days after immunisation showed a decrease in proliferative responses to the immunising cell line with an increase in proliferative responses to *T. annulata* (auto.) cell line. Responses to the autologous cell line and IL-2 were at their peak on day 16 after immunisation.

#### **4.4.2 DNA flow cytometric analysis of the DLN cells**

A quiescent cell which is not dividing or growing is often referred to as being in the  $G_0$  state of the cell cycle. When the cell starts to make new RNA, it has entered the  $G_1$  phase where certain proteins essential for replication of DNA are made. After  $G_1$ , the cell enters into the S phase where the cell starts to make new DNA. Once the amount of DNA has doubled, cell enters the  $G_2$  and finally in mitosis (M) phase. Cells in the  $G_2$ /M phase have double the amount of DNA compared with those as in the  $G_0$ / $G_1$  phase. The DNA content of S phase will be between these two extremes (Ormerod, 1994).

The percentage of the total nuclei, excluding the hypodiploid nuclei, within the S phase of the cell cycle was determined by S-Fit analysis as shown in Table 4.1 and Fig. 4.5. A large number of cells had entered into the S-phase of the cell cycle within 4 days after *T. annulata* cell line immunisation. The increase was >100% as compared to the naive cells. About 75% more cells entered into the S-phase by day 9 after immunisation as compared to cells of the naive calf with 50% more in  $G_2$ /M as compared to day 4. 50% more cells were observed in the S-phase of cell cycle on

**Table 4.1**  
**Percent population of cells in different phases of cell cycle**

Day (Calf)	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M
Day 0 (Naive)	80	8.6	10.9
Day 4 (12848)	73.5	18.8	7.7
Day 9 (12810)	73	14	11
day 12 (12851)	76	10	12
Day 16 (12852)	74	12	13

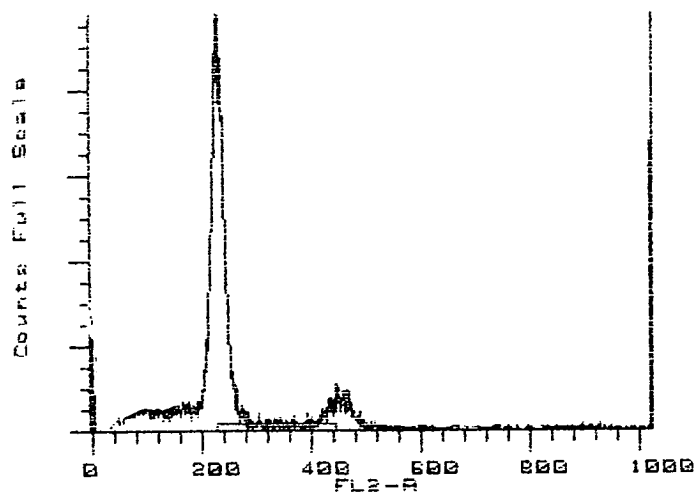
**Table 4.2**  
**Percent population of apoptotic cells**

Days	Day 0	Day 4	Day 9	day 12	Day 16
% Apoptotic cells	4.4	4.5	7.14	5.4	5.2



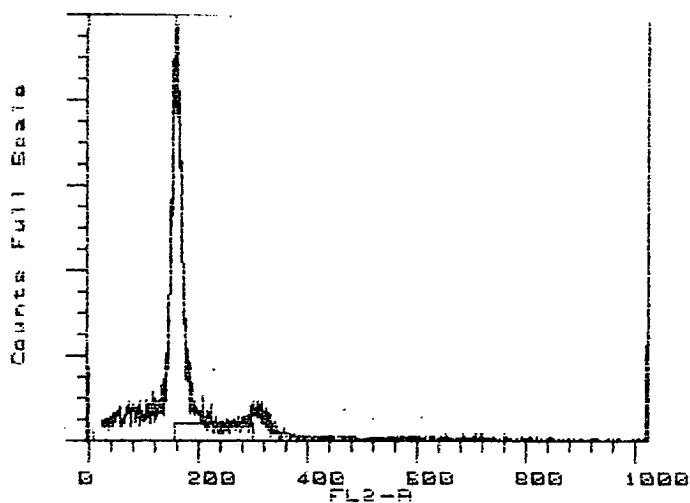
Naive  
S  
8.6%

G<sub>2</sub> / M  
10.9%



Day 4  
S  
18.8%

G<sub>2</sub> / M  
7.7%



Day 16  
S  
12%

G<sub>2</sub> / M  
13%

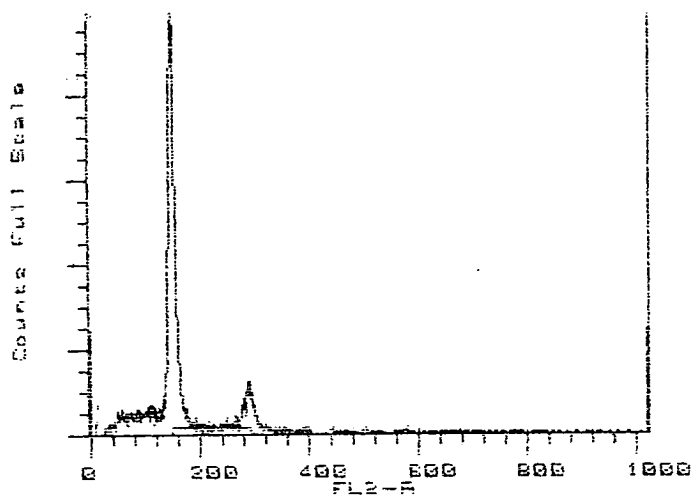


Fig. 4.5 Number of DLN cells in S and G<sub>2</sub>M phases of cell cycle in naive, day 4 and day 16 after immunisation.

day 16 after immunisation as compared to cells of naive calf. A maximum of 13% occurred in the G<sub>2</sub>/M phase.

The percentage of cells within the Sub-G<sub>1</sub> peaks are detailed in Table 4.2. A total of 4.4% cells were apoptotic in the DLN of the naive animal. A total of 7.14% of apoptotic cells were observed on day 9 after immunisation as compared to the naive. The number of apoptotic cells returned to base level by day 16 after immunisation.

#### **4.4.3 Immunoperoxidase test for antibodies**

Antischizont antibody titers were measured by an immunoperoxidase test. The highest titer produced was 1:10240 in calf 12852 (node removed on day 16) in the 4th week after immunisation with a peak titer of 1:2560 in calf 12851 (node removed on day 12). This peak was maintained until the 4th month after immunisation, antibody titers later declined by 6th month to 1:640 and 1:160 in calves 12852 and 12851 respectively.

### **4.5 DISCUSSION**

The results in this chapter are discussed in the context of the findings described in chapter 3. There was febrile reaction in all the four animals during the first 10 days after immunisation, a decline in TLC and TEC, an increase in the number of lymphocytes in the medulla by day 9 (chapter 3). These changes were thought to be related to the calves' immune responses to the MHC antigens of the immunising cell line. This suggestion was further confirmed in this chapter, when the DLN cells were shown to proliferate strongly in response to antigens of the allogeneic immunising cell line but weakly in response to the parasitised autologous cell line. Similar results have been obtained in studies on efferent cells draining the lymph

node (Nichani, 1995). The proliferation of the DLN cells in response to the IL-2 confirmed the activated state of the T cells and this correlated well with finding IL-2R<sup>+</sup> cells in lymph node sections prepared on day 4 after immunisation (chapter 3).

The second peak of temperature during the 3rd week of immunisation with the drop in PCV looked like a parasite specific immune response (chapter 3). This suggestion was confirmed when the DLN cells were found to proliferate strongly in response to the autologous cell line and to rIL-2. They reflected previous findings that the maximum proliferation of efferent lymph cells occurred in response to the autologous cell line after day 12 (Nichani, 1995). Nichani further noticed that such proliferative responses to recall antigens were associated only with the animals exhibiting mild parasitological reactions after immunisation. Similar antigen specific cells in *Toxoplasma gondii* infection were observed after *in vitro* stimulation with the antigen (Innes *et al*, 1995).

Although, no cytotoxicity assays were performed in the present study, it seems likely that similar results would have been obtained as were seen with cells from the efferent lymph from the draining node after immunisation of calves with 10<sup>6</sup> cells of a *T. annulata* (H) 10769 cell line (the same cell line at the similar cell concentration was used in the present study) (Nichani,1995). Nichani reported cytotoxic responses against the MHC antigens of the immunising cell line which started at around day 5-6 and reached its peak by days 7-10. In the parasite specific response, which peaked by days 15-18, cytotoxicity was against the autologous macroschizont-infected cells only. Similar findings have been reported in PBM after *T. annulata* allogeneic cell line immunisation (Innes *et al*, 1989a) and after *Theileria parva* allogeneic cell line immunisation (Emery *et al*, 1982).

A 100% increase in the number of cells in the S phase of the cell cycle was observed by flow cytometry on day 4 after immunisation as compared to day 0. This change was about 75% by day 9 after immunisation. The presence of 50% more cells in S phase and maximum in G<sub>2</sub>/M phase by day 16 could have been due to parasite specific responses. These findings correlated well with the presence of a large number of MIB-1<sup>+</sup> cells in the paracortex and medulla of the DLN (Chapter 3) and with the presence of blasting cells in the lymph efferent from the node (Nichani, 1995). Two peaks of blasting cells were reported. The first was between days 6-11 and was associated with the development of an allogeneic response against the MHC antigens of the immunising cell line. The second increase was from day 13 onwards and was reported to be associated with the parasite specific immune responses. Both these phases of cell cycling correlate well with the findings of *in vitro* proliferation assays. In contrast, the presence of large numbers of cells in the S-phase in lymph nodes of calves undergoing lethal sporozoite induced infection appeared to be due to proliferation of the parasite infected cell (Campbell, 1995).

About 7% apoptotic cells were recorded by day 9 after immunisation. Although the number of apoptotic cells was more than in naive calves, it was not as high as the 22% observed in the DLN of calves undergoing lethal sporozoite induced infection (Campbell, 1995). These results indicated the mild infection induced by cell lines described here did not stimulate cells to undergo apoptosis whereas lethal sporozoite induced infection did.

The anti-schizont antibody titer of 1:2560 and 1:10240 in the animals 12851 and 12852 respectively correlated with the appearance of large number of well developed

GC. The light zone of the GC is the site for the selection and isotype switching of the B cells with the rescue signal of T<sub>H</sub> cells. CD3<sup>+</sup> cells were found in the light zone of GC by day 16 with the appearance of VPM30<sup>+</sup> B cells (chapter 3), this may have indicated role for these CD3<sup>+</sup> T cells. As far as role of these antibodies is concerned, the literature points towards their nonprotective role (Dhar *et al*, 1978; Samad *et al*, 1984). In *in vitro* blocking assays, immune serum prevented the infection of cells by sporozoites of *T. annulata* (Ahmed *et al*, 1988; Gray & Brown, 1981; Preston & Brown, 1985) whereas the immune serum could not inhibit the proliferation or lyse either *T. annulata* schizont infected cells (Preston & Brown, 1985; Ahmed *et al*, 1988; Shiels *et al*, 1989) or *T. parva* infected lymphocytes (Creemers, 1983). From all the above studies, it looks as if the antibody responses are not protective against the schizont stage of *Theileria* but appear to be protective against *Theileria* sporozoites. In contrast, calves immunised with an allogeneic cell line showed macrophage mediated antibody dependent cell cytotoxicity against the macroschizont-infected cells (Preston & Brown, 1988).

In summary, by immunohistology and functional assays, two phases of the immune response, first against the allogeneic antigens of the immunising line and later to the parasite were observed in the DLN in response to immunisation with an allogeneic cell line. As T cell mediated immune responses develop in the DLN, it seems likely that these activated T cells may be producing some cytokines. For example, very high IFN $\gamma$  production was reported recently in the efferent lymph (Nichani, 1995) and DLN after sporozoite infection (Campbell *et al*, 1995). IFN $\gamma$  has been shown to provide the T<sub>H1</sub> type of response needed to control intracellular parasites (Murray *et al*, 1983; Scott *et al*, 1988, Scharton *et al*, 1993; Sher *et al*, 1993).

These observations raised questions as to the nature of the cytokines produced in

response to immunisation with cell lines and to their role in immune responses *in vivo*. For inoculation with cell lines led to self-healing infections which indicated  $T_{H2}$  and  $T_{H1}$  cytokines were acting in a critically balanced manner. Otherwise excessive or inappropriate production of either would have led to disease rather than protective immunity as shown in infection with *T. gondii* (Roberts *et al*, 1996). The work observed in the following chapter investigated the cytokine profiles of the DLN cells in calves immunised with cell lines.

## **CHAPTER 5**

### **KINETICS OF CYTOKINE PRODUCTION BY DRAINING LYMPH NODE CELLS FOLLOWING IMMUNISATION WITH *THEILERIA ANNULATA* INFECTED CELL LINES**

## 5.1 INTRODUCTION

Various T cell cytokines appear to contribute to the development of immunity or infection in mice, human and animals. The production of IFN $\gamma$  has been related to disease resistance in certain circumstances (Gaffar *et al*, 1995; Scott *et al*, 1988; Suzuki *et al*, 1988; 1989). In other circumstances, a high production of IFN $\gamma$  has been shown to promote infection (McLeod *et al*, 1989; Roberts *et al*, 1996).

Some evidence exists for a role for IFN $\gamma$  in *T. annulata* infection in cattle. Bovine recombinant IFN $\gamma$  in cattle, in *in vitro* studies, inhibited the establishment and transformation of trophozoite-infected cells (Preston *et al*, 1992b). PBM from lethally infected cattle spontaneously produced IFN $\gamma$  (Preston *et al*, 1993). In a recent, *in vitro* study, the addition of rIFN $\gamma$  had an inhibitory effect on the multiplication of *T. annulata* (macroschizont) infected cell lines as did efferent lymph rich in IFN $\gamma$  obtained from calves immunised with cell lines (Nichani, 1995).

In *in vivo* studies following *T. annulata* sporozoite infection, Nichani (1995) demonstrated a very high level of IFN $\gamma$  production in efferent lymph and it has been proposed that IFN $\gamma$  may promote the pathology of tropical theileriosis rather than resistance to *T. annulata* infection (Preston *et al*, 1993; Campbell, 1995). Finding 20 times more IFN $\gamma$  in efferent lymph of lethally infected animals than in cell line immunised animals (Nichani, 1995) supports this view. While IL-4 appears to downregulate the production of IFN $\gamma$  and thereby prevent mortality in toxoplasmosis (Roberts *et al*, 1996), nothing is known about its role in cattle immunised with *T. annulata* infected cell lines.

The experiments described in this chapter were designed to examine the cytokine



profile of the DLN, in particular IL-4 and IFN $\gamma$ . IL-2 and IL-2R were also examined as indicators of T cell activation.

## **5.2 EXPERIMENTAL DESIGN**

RT-PCR was used to monitor cytokine mRNA in the lymph nodes draining the site of inoculation of a *T. annulata* (Hisar, 10769) cell line, which had already been examined by histological and immunological methods (chapter 3 & 4). RNA was isolated from the frozen DLN cells as well as from the frozen DLN tissue of calves (13046, 12848, 12810, 12851 & 12852). T<sub>H1</sub> cytokines (IL-2, IL-2R & IFN $\gamma$ ) and T<sub>H2</sub> cytokine (IL-4) mRNA were assessed.

## **5.3 MATERIALS AND METHODS**

RNA of DLN was isolated and reverse transcribed into cDNA. This cDNA was amplified by PCR using the cytokine primers and the amplified PCR products analysed by gel electrophoresis.

### **5.3.1 Isolation of RNA**

#### **5.3.1.1 Isolation of RNA from frozen DLN cells**

RNA was isolated using a commercial kit, "RNeasy total RNA" (Quiagen, Germany). This system is based upon purification columns and was chosen because it is faster than other methods. Briefly, frozen cells were thawed quickly, washed twice with 10ml RPMI and centrifuged for 10 min. @ 1000 RPM. The 10<sup>7</sup> cells were washed with PBS in an 1.5 ml eppendorf centrifuge tube by centrifuging at 1000 RPM for 5 min. The supernatant was removed by drying on a tissue paper. 350 $\mu$ l of lysis buffer RLT (provided with the kit) with mercaptoethanol was added to the cell pellet which was homogenised by passing through a syringe fitted with a 23# needle. An

equal volume of 70% ethanol was added to the solution which was applied to the RNeasy spin column. The column was centrifuged at 10,000 RPM for 15 sec. and the supernatant was discarded. The column was then washed with 700µl of RW1 wash buffer (provided with the kit) by centrifuging at 10,000 RPM for 15 sec. The liquid along with the collection tube was discarded. The column was transferred to a fresh collection tube and was washed with 500µl RPE wash buffer at 10,000 RPM for 15 sec. It was washed again with 500µl RPE wash buffer by centrifuging at 15,000 RPM for 2 min. to make the column completely dry. The column was then transferred to a new collection tube and 50µl of sterile distilled water was added to it. The column was kept on ice for 5 min. and then centrifuged at 10,000 RPM for 1 min. to collect the RNA. The RNA was quantified using a spectrophotometer as described below.

#### **5.3.1.2 Isolation of RNA from the DLN tissue**

About 150mg tissue was chopped in 1.75ml of RLT lysis buffer (Quiagen) with mercaptoethanol. The tissue was chopped using sterile scalpels and homogenised in a sterile glass homogeniser. This lysate was further homogenised by passing through the syringe fitted with a 23# needle several times before applying 350µl of this homogenised solution to the spin column. 350µl of 70% ethanol was added to the column. The rest of the procedure was same as described above.

#### **5.3.2 Quantification and purity of RNA**

The concentration of RNA was determined by spectrophotometry as described by Sambrook *et al*, (1989). The spectrophotometer was calibrated with distilled water at absorbance 260nm and 280nm. The RNA was diluted 1:100 with sterile distilled water before taking readings. Concentration of the RNA was calculated as follow: An absorbance reading of 1 at 260nm was taken to correspond to 40µg/ml RNA.

Concentration of RNA=  $40 \times A_{260} \times 100$  (Dilution factor)= RNA  $\mu\text{g}/\text{ml}$

Total concentration in 50  $\mu\text{l}$ = RNA  $\mu\text{g}/\mu\text{l}$   $\times 50$

The purity of the RNA was assessed from the ratio of  $A_{260}$  to  $A_{280}$ , where  $A_{280}$  is the maximum absorbance for proteins. Ideally the ratio of  $A_{260}$  to  $A_{280}$  will be 1.8-2. RNA was aliquoted and stored at  $-80^{\circ}\text{C}$  till used.

### **5.3.3 cDNA synthesis**

The first strand cDNA synthesis was carried out by reverse transcription using the superscript system (Life Technologies Ltd.) as per maker's instructions. 5  $\mu\text{g}$  of RNA was used to synthesise cDNA. It was made up to 12  $\mu\text{l}$  in sterile water and the reaction was primed with 1  $\mu\text{l}$  of 500  $\mu\text{g}/\text{ml}$  oligo dT primer and incubated at  $70^{\circ}\text{C}$  for 10 min using a Hybaid, OmniGene thermal cycler (PCR machine). It was quickly chilled on ice for 5 min. This mixture with the oligo dT primer bound to the polyA tail of the mRNA was reverse transcribed to make cDNA. This 12  $\mu\text{l}$  mix was mixed with 4  $\mu\text{l}$  10x synthesis buffer, 2  $\mu\text{l}$  0.1M dithiothreitol (DTT), 1  $\mu\text{l}$  100mM dNTP mixture and 1  $\mu\text{l}$  200U/ $\mu\text{l}$  reverse transcriptase (superscript). It was mixed well, kept at room temp. for 10 min. and incubated at  $42^{\circ}\text{C}$  for 50 min. followed by heating at  $95^{\circ}\text{C}$  for 5 min. to terminate the reaction. It was chilled on ice for 5 min. The cDNA could either be used immediately in a PCR for amplification or stored at  $-20^{\circ}\text{C}$  till used.

### **5.3.4 Amplification of expressed cytokine mRNA**

Amplification of cytokine mRNA was undertaken using the primers listed in Table 5.1.

**Table 5.1      Primer Sequences**

mRNA	Strand	T°C	Size bp	5'-Sequence-3'
G3PD	+	57	565	GATGCTGGTGCTGAGTATGTAGTG
	-	57		ATCCACAACAGACAGCTTGGGAG
IL-2	+	51	255	AAGTCATTGCTGCTGGATTAC
	-	52		CCTGTAGTTCCAAAACGATTCTC
IL-2R	+	63	529	GCCAACAAGAGGCTGAAAGGAAACC
	-	63		TGCCCCAGCGTGAAATGGTAGAC
IL-4	+	58	457	GCATTGTTAGCGTCTCCTGGTAAAC
	-	55		CTTCATAATCGTCTTTAGCCTTTCC
IFN $\gamma$	+	59	561	GGAGCTACCGATTTCAACTACTCCG
	-	59		GCAGGCAGGAGGACCATTACG

+ Coding sequence

- Non coding sequence

#### 5.3.4.1 *Primer design*

The primers were designed from the published RNA or DNA sequences by using PCRPLAN PC/GENE release 6.7 software (IntelliGenetics Inc., Mountain View, California). Primers for the amplification of the cytokine products were designed from the published sequences of bovine IL-2, IL-2R (p55 sub unit), IL-4 and IFN $\gamma$  (Reeves *et al*, 1986; Weinberg *et al*, 1988; Heussler *et al*, 1992; Cerretti *et al*, 1986 respectively). Primer sequences, predicted melting temperature (T) and fragments sizes are listed in Table 5.1. The primer of bovine G3PD was included throughout the study as a positive control as it is an essential enzyme in glycolysis (Stryer, 1981). The G3PD primer sequences were kindly provided by the ILRI, Kenya. Primers were designed by Drs. Dougie Fraser and George Russell.

#### 5.3.4.2 *RT-PCR*

PCR amplification from cDNA was carried out as described by Innis and Gelfand (1990). The same amount of RNA (5 $\mu$ g) was used in each cDNA preparation and 4 $\mu$ l (1 $\mu$ g RNA) of cDNA reaction mixture was used in each PCR reaction mixture. PCR was carried out in 50 $\mu$ l reaction mixtures which were comprised of

5 $\mu$ l 10X PCR buffer (Gibco)

2 $\mu$ l 50mM MgCl<sub>2</sub> (Gibco)

1 $\mu$ l 25  $\rho$ M + primer

1 $\mu$ l 25  $\rho$ M - primer

4 $\mu$ l cDNA

1 $\mu$ l 10mM dATP (Gibco)

1 $\mu$ l 10mM dCTP (Gibco)

1 $\mu$ l 10mM dGTP (Gibco)

1 $\mu$ l 10mM dTTP (Gibco)

.4µl Taq DNA polymerase 2U( Gibco)

32.6µl Distilled water

Control samples were also included which contained primers but no cDNA to ensure that the detection of products was not due to environmental contamination. As the amount of RNA and the cDNA used was the same for all the samples, a degree of comparison was possible between the assays.

The above reaction mixtures were amplified using a Hybaid Omne Gene thermal cycler. The predicted temperature for the primers ranged from 51°C to 61°C. Since these primers have already been used at 55°C in this laboratory (Brown *et al*, 1995; Campbell 1995), they were used at 55°C in this study. The samples were heated to 95°C for 5 min. to ensure complete denaturation of RNA/DNA complexes followed by amplification as follows: 95°C for 1 min.(denaturation), 55°C for 1 min. (primer annealing), 72°C for 1 min. (primer extension). A total of 30 cycles were run for the amplification of all the mixtures. A final incubation at 72°C for 5 min. was carried out to ensure complete extension of products.

Following amplification, PCR products were electrophoresed immediately by gel electrophoresis at 100V, using TBE as the running buffer in 2% agarose gels. 1µl of loading buffer was added per 10µl of PCR products (sample) analysed. HaeIII size marker (Sigma) was used throughout the experiments. HaeIII markers consists of fragments which forms three blocks of bands as follows:

587/540/504/458/434

267/234/213/192/184

124/123/104/89/80/64/57/51/21/18/11/8

## 5.4 RESULTS

The cytokine mRNA profiles of the lymph node of a naive calf (Fig. 5.1) and of the draining lymph nodes of immunised calves at 4, 9, 12 and day 16 post immunisation as detected by RT-PCR are summarised in Table 5.2 & shown in Fig. 5.2 to Fig. 5.5. mRNA of IL-4 and IFN $\gamma$  were very faint in the naive node (Fig. 5.1). By day 4 after immunisation a complete absence of IL-4 with a strong IFN $\gamma$  was detected (Fig. 5.2, a band at the bottom is that of primer). IFN $\gamma$  became very faint with the continued absence of IL-4 by day 9 post immunisation (Fig. 5.3). A total absence of IFN $\gamma$  with a continued absence of IL-4 was detected by day 12 post immunisation (Fig. 5.4). Reappearance of very faint products of IL-4 with faint product of IFN $\gamma$  was observed by day 16 of immunisation (Fig. 5.5). The mRNA bands for IL-2 was strong and IL-2R was very weak in naive calf but IL-2R became very strong on day 4 after immunisation (Table 5.2 and Fig. 5.1 - 5.5).

## 5.5 DISCUSSION

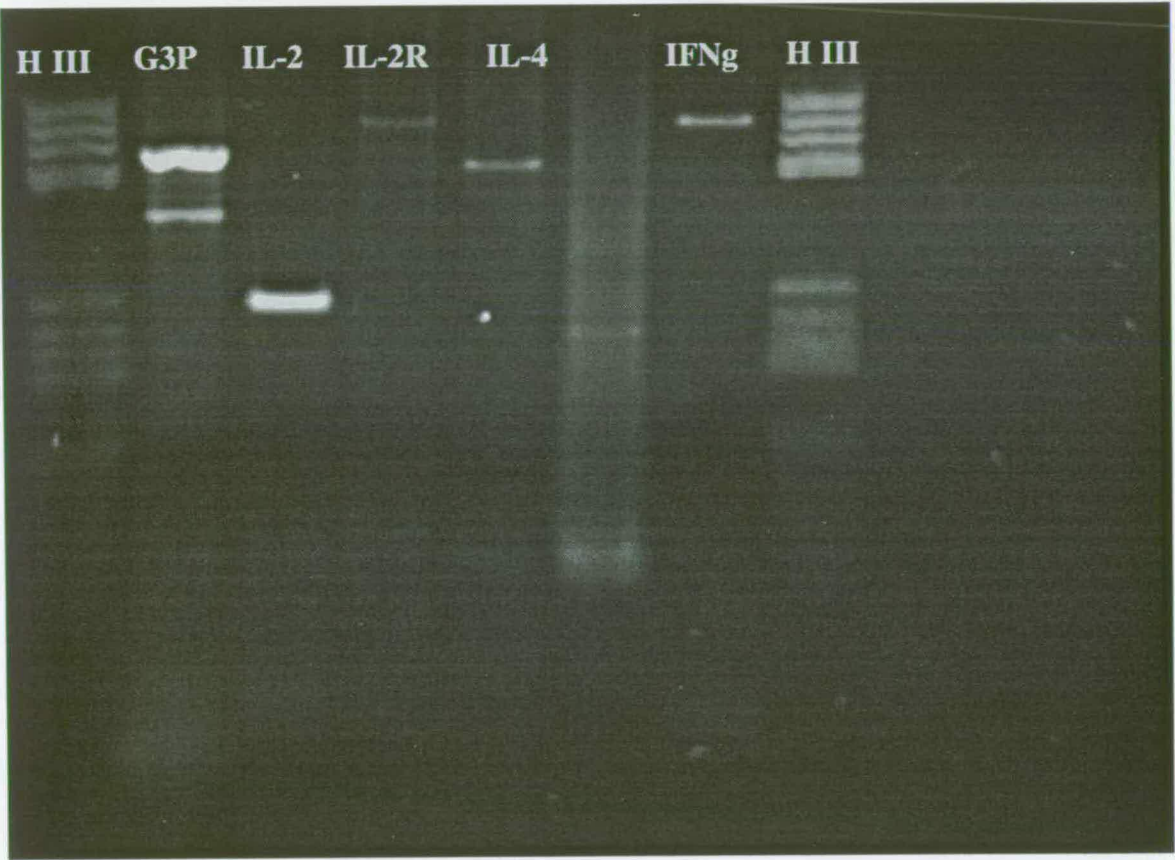
In the present study, preliminary RT-PCR studies were performed on DLN cells to identify the different cytokine mRNA expressed in naive calves and calves immunised with a *T. annulata* cell line. DLN cells of immunised animals were examined on days 4, 9, 12 and 16 after immunisation. As the GC formation and the development of B cells is dependent upon T cells, T cells have been studied extensively for their ability to produce cytokines after *in vitro* stimulation. For example, The *in vitro* proliferation of B cells can be supported by cytokines like IL-4 (Defrance *et al*, 1987) and IFN $\gamma$  (Romagnani *et al*, 1986). However, the results of cytokines obtained after *in vitro* stimulation do not always reflect *in vivo* results and very little is known about *in vivo* cytokines production of GC T cells even though the T cells are essentially critical for GC formation (Jacobson *et al*, 1974) and are believed to be antigen specific (Kathleen

**Table 5.2**  
**Cytokine mRNA detected in DLN on days 0, 4, 9, 12, and 16 after immunisation with *T. annulata* cell lines.**

Day (Calf)	IL-2	IL-2R	IL-4	IFN $\gamma$
0 (Naive calf)	++	+/-	+/-	+/-
4 (Calf 12848)	+	+++	-	++
9 (Calf 12810)	+++	++	-	+/-
12 (Calf 12851)	+/-	++	-	-
16 (Calf 12852)	+++	+	+/-	+/-

+ refers to positive mRNA band  
- refers to negative mRNA band  
+/- refers to weak mRNA band  
++ strong mRNA band  
+++ very strong mRNA band





**Fig. 5.1** Naive lymph node cytokine profile. See mRNA bands for IL-2, IL-2R, IL-4 and IFN $\gamma$ .

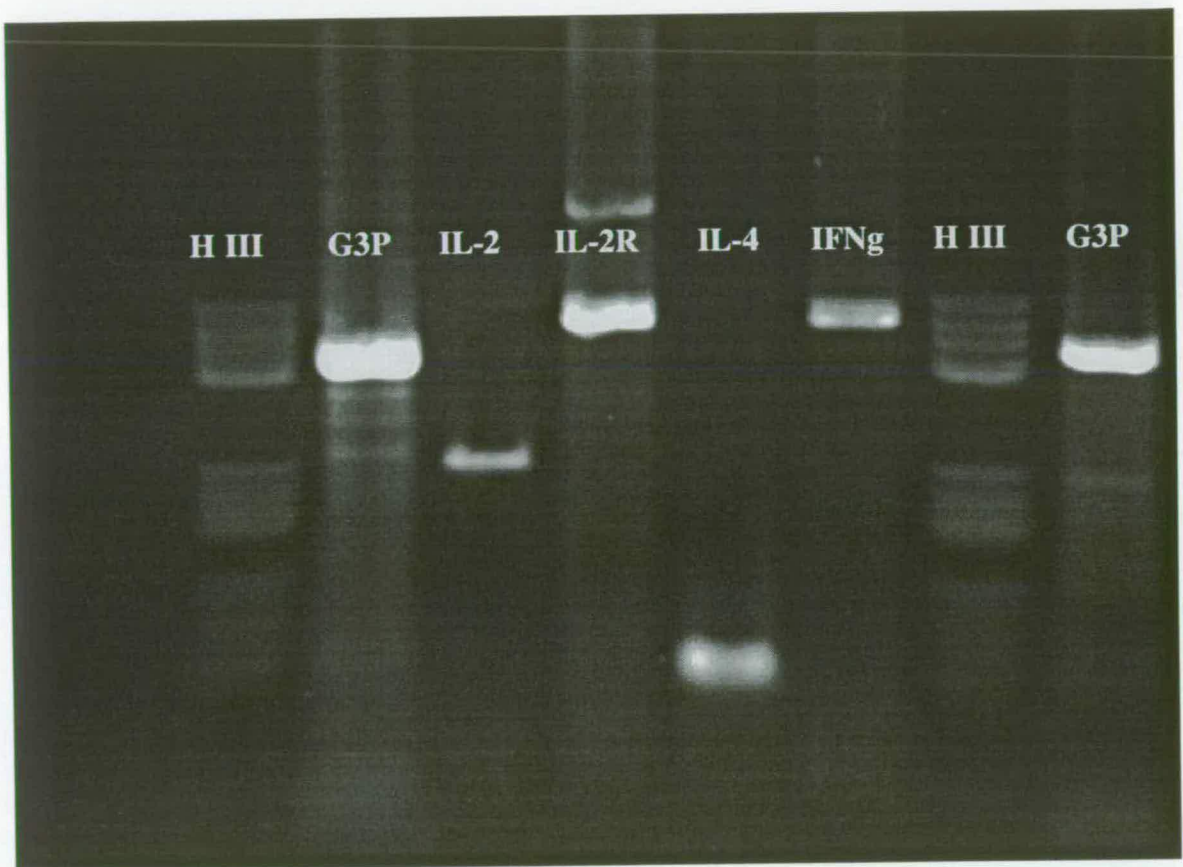


Fig. 5.2 Draining Lymph node cytokine profile 4 days after immunisation with *T. annulata* cell line. See mRNA band for IL-4 has been lost and IFN $\gamma$  has become strong.

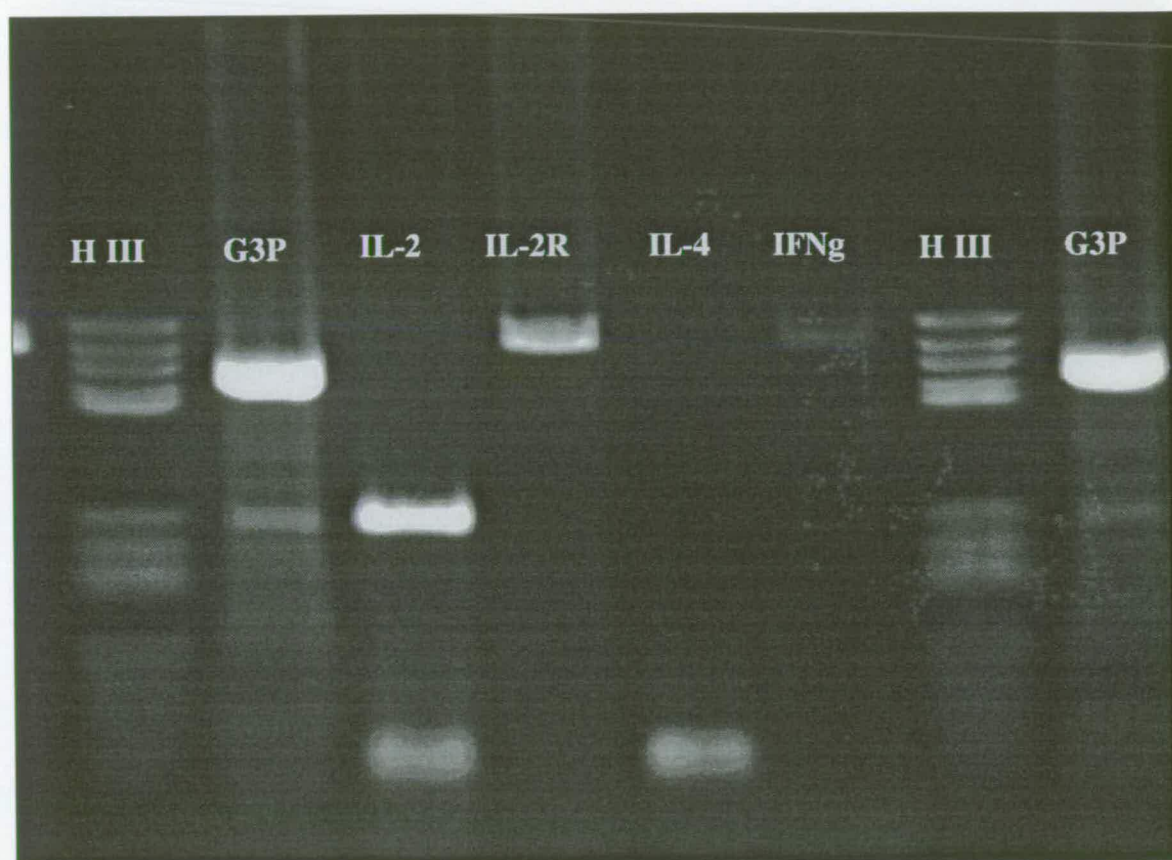


Fig. 5.3 Draining Lymph node cytokine profile 9 days after immunisation with *T. annulata* cell line. The mRNA band for IFN $\gamma$  has become weak as compared to Fig. 5.2

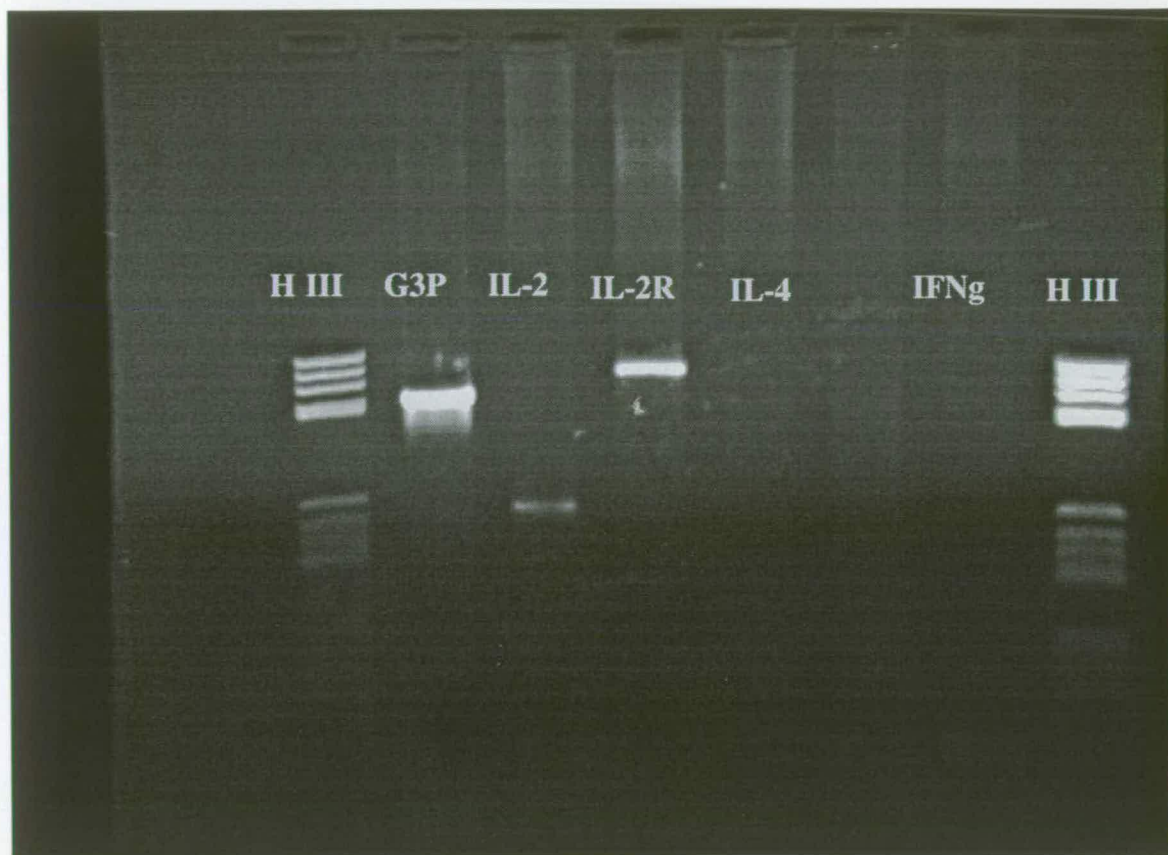


Fig. 5.4 Draining Lymph node cytokine profile 12 days after immunisation with *T. annulata* cell line. mRNA for only IL-2 and IL-2R has been detected.



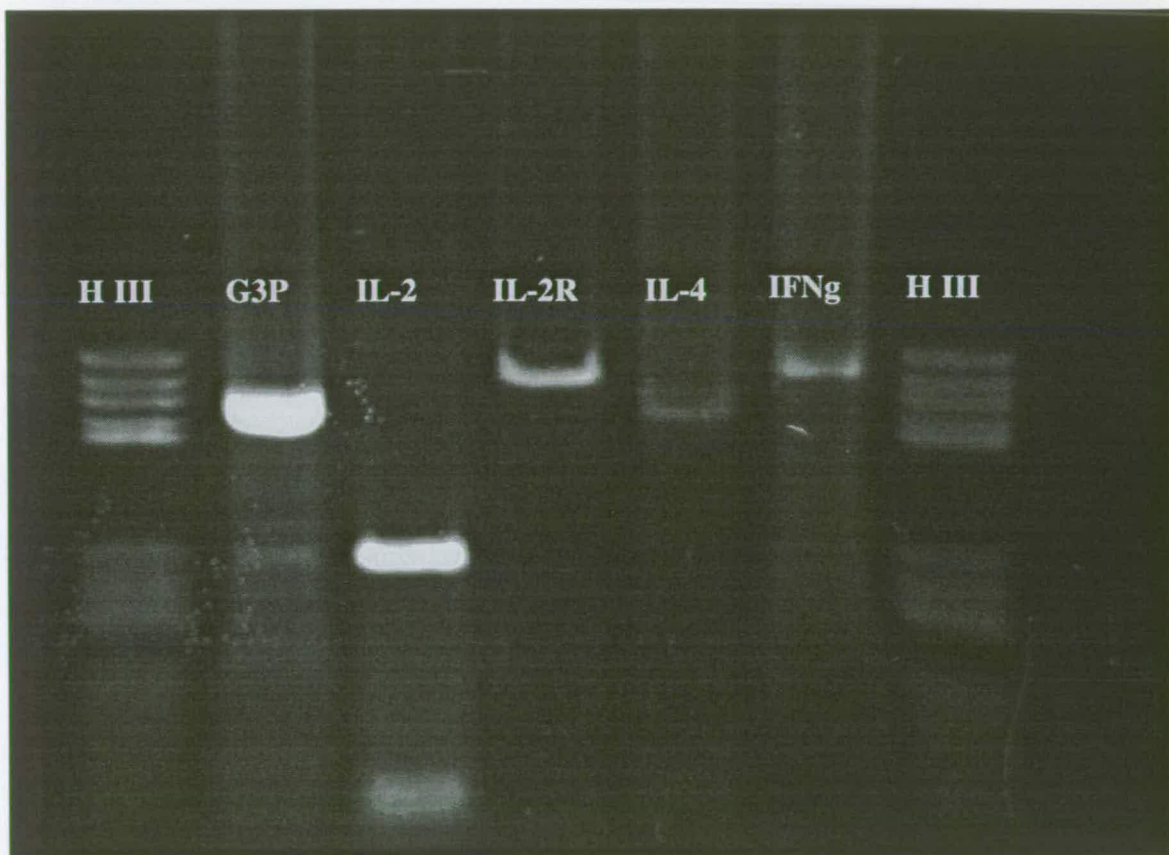


Fig. 5.5 Draining lymph node cytokine profile 16 days after immunisation with *T. annulata* cell line. The mRNA for IL-4 and IFN $\gamma$  has reappeared.

*et al* 1993). Our findings that when the number of T cells in the primary follicles increased with the appearance of GC agrees with these observations (chapter3.4.4.1).

The study of Butch *et al*, (1993) showed that GC T<sub>H</sub> cells and not the non GC T<sub>H</sub> cells consistently produced IL-4. We observed T cells and B cells in close association with each other in the PF/GC (chapter 3) which suggested a role for IL-4 in GC B cell development and isotype switching. The initialisation of dark zone proliferation appears to be a stage of B cell development. The induction of B cell proliferation and initiation of GC before immunoglobulin production is believed to be cytokine independent. For example T<sub>H2</sub> cytokines like IL-4, play a role in Ig secretions only after proliferation (Hodgkins *et al*, 1991). Similar findings were observed in this study. The presence of a small number of T cells with a small number of MIB-1<sup>+</sup> proliferating cells in the dark zone of PF/GC up to day 12 (chapter3.4.4.1) with the absence of IL-4 was observed (this chapter). Presence of a large number of T cells in the light zone (chapter 3), with the expression of mRNA IL-4, in close proximity to VPM 30<sup>+</sup> B cells by day 16 correlates with the findings of Hodgkins (1991) that this later process is IL-4 dependent. The mAb VPM30 appears to recognise a differentiation antigen acquired by B cells upon passage to the light zone. The expression of Ag recognised by VPM30 on B cells is proposed to be dependent upon T cell help (Campbell, 1995) and this above study strongly indicated a role for IL-4 for the expression of VPM30<sup>+</sup> cells in the light zone.

Extremely high level of IFN $\gamma$ , 80-120ng/ml, in the lymph efferent from the draining node (Nichani, 1995) and IFN $\gamma$  mRNA in the DLN cells (Campbell, 1995) after *T. annulata* sporozoite infection were believed to be related to the severity of the disease. With the loss of IL-4, total destruction of the GCs were reported. All the

animals in above studies suffered acute theileriosis and had to be treated to save their lives. As compared to very high level of IFN $\gamma$  in the efferent lymph after *T. annulata* sporozoite infection, a very low level was found in the efferent lymph after *T. annulata* cell line immunisation (Nichani, 1995). Early IFN $\gamma$  production during DLN responses has been suggested to influence subsequent T cell activation (Bogen *et al*, 1993). IFN $\gamma$  is a strong antagonist to IL-4 producing cells (Donckier *et al*, 1994). Apparently, a similar mechanism is in operation early after immunisation. In this study, strong mRNA for IFN $\gamma$  was observed with the loss of IL-4 on day 4. By day 9, the mRNA for IFN $\gamma$  was weak with the total loss by day 12. Both IL-4 and IFN $\gamma$  reappeared by day 16 after immunisation. This interplay of IL-4 and IFN $\gamma$  is crucial for the outcome of any infection or immunisation (Roberts *et al*, 1996). This appears to be true as in *T. annulata* infection, Campbell (1995) reported a total loss of mRNA for IL-4 with the early production of IFN $\gamma$  in draining lymph nodes in animals suffering from acute theileriosis.

In another protozoan parasite, *Toxoplasma gondii*, IFN $\gamma$  has been related to its protective role in infection (Suzuki *et al*, 1988 and 1989) along with the highest mortality reported in mice producing highest level of IFN $\gamma$  (McLeod *et al*, 1989). Similarly, in a separate study, IL-4 knock out mice produced high IFN $\gamma$  early in infection and suffered more than immunocompetent animals (Roberts *et al*, 1996). Both these studies suggest high IFN $\gamma$  as an aid to parasite proliferation specifically in the absence of IL-4.

IL-4 has earlier been shown to suppress TNF $\alpha$  production directly (Essner *et al*, 1989). IFN $\gamma$  along with TNF $\alpha$  activates macrophages to produce reactive oxygen (Hughes *et al*, 1988) and nitrogen intermediates (Sibley *et al*, 1991), both of which

have been shown to control protozoan parasites (Wright *et al*, 1988; Liew & Cox, 1991; Jun *et al*, 1993; Peterson *et al*, 1995). However both of these products have toxic effects if produced in excess (Clark *et al*, 1991). Similar findings have been reported in the *in vitro* studies where nitric oxide (NO) has a cytostatic effect on cells infected with *T. annulata* trophozoites or macroschizonts (Visser *et al*, 1995a). This suggests that NO might help in the control of parasite development and be a causative agent of disease, if produced in excess, because of its toxic effects (Visser *et al*, 1995b). Findings in this study and the observations in the literature strongly suggest the production of IFN $\gamma$  is controlled by IL-4 thereby preventing its direct inflammatory effect.

The production of IL-2 and IL-2R by day 4 indicated the activated state of T cells ( $T_{H1}$  response) as shown immunohistologically by the presence of IL-2R<sup>+</sup> and MIB-1<sup>+</sup> cells in the paracortex. This also correlates well with our findings in chapter 4, that the DLN cells by day 4 and 9 proliferated well in response to the allogeneic immunising cell line. As IL-2 and IL-2R are essential for CTLs, it is possible that a CTL response might be helpful in the release of the macroschizont from the immunising cell line into the lymph node. The presence of IL-2 and IL-2R throughout the period confirmed the activated state of T cells. These findings correlated well with the results of proliferation assays (chapter 4) in which DLN cells proliferated well in response to exogenous rIL-2.

We were surprised by the weakness of band of IL-4 on day 16 as hyperplastic GC (chapter 3) with peak antibody titers (chapter 4) were recorded on day 16 after immunisation. This may have been downregulated by IFN $\gamma$ .



The question is to whether the initial responses to cell lines occur in the DLN or at the site of immunisation was investigated by studies using afferent cannulation described in the next chapter.

To summarise, *T. annulata* cell line stimulated T cells in the DLN with the production of mRNA for IL-2, IL-2R, IFN $\gamma$  and IL-4. The sequential production of IFN $\gamma$  and IL-4 seemed to be delicately balanced as inappropriate or excessive production of either could have caused pathology in the DLN.

## **CHAPTER 6**

**A NOVEL METHOD OF CANNULATION FOR COLLECTING THE  
PSEUDOAFFERENT LYMPH OF CATTLE FOLLOWING  
IMMUNISATION WITH *THEILERIA ANNULATA* INFECTED CELL**

**LINES:**

**ANALYSIS OF PARASITE TRANSFER AND DISSEMINATION**

## 6.1 INTRODUCTION

Immunisation with a *T. annulata* infected allogeneic cell line initially produced an anti-MHC immune response against the allogeneic immunising cell line followed by a parasite specific immune response in the DLN (Chapter 3, 4 & 5) as reported previously in PBM and efferent lymph of cattle (Innes *et al*, 1989a; Nichani, 1995). The transfer of macroschizonts from the cells of the vaccine to the cells of the immunised animal is considered essential for successful immunisation with cell lines (Brown *et al*, 1978a; Innes *et al*, 1989a). Thus in a recent study, the recipient's cell line could not be isolated from one of the immunised animals and that animal suffered with a disease upon challenge (Nichani, 1995) presumably due to the failure of the parasite to transfer to and proliferate sufficiently in the recipients cells to stimulate immunity. However, nothing is yet known about the site of parasite transfer and little is known about dissemination of the parasite from the site of immunisation.

Studies of parasite transfer and dissemination necessitated the collection of afferent lymph by cannulation of the pseudoafferent lymphatics. Cattle have been cannulated before (Emery *et al*, 1987) but the animals have had to be restrained in a crush thereby restricting their free movement as well as possibly access to food and water. As the animals were restrained, and hence to some degree under stress, restraint itself may have affected the results.

A novel method of pseudoafferent cannulation was required to achieve the objectives, without putting the animals under unnecessary stress. This study was undertaken therefore:

- i) To develop a new method of cannulation which did not require cattle to be permanently restrained.

ii) To use this method to investigate site of parasite transfer and its dissemination from the site of immunisation.

## **6.2 EXPERIMENTAL DESIGN**

### **6.2.1 The novel method of cannulation**

The existing method of pseudoafferent cannulation used a double lumen cannula with the animal restrained in a crush. The anticoagulant being pumped down one lumen and lymph collected from the other. Both the pump and collecting bottle were placed outside the crush away from the animal.

The novel development devised here was to attach a light weight low speed infusor and the collecting bottle to a collar around the animal's neck and to cover the whole with the expanding netting. The animal was then free to move about at will and its stress reduced. All surgery was performed by Dr. Roger Spooner.

### **6.2.2 Parasite transfer and its dissemination**

The pseudoafferent ducts of young calves were cannulated (as above) and the animals immunised with *T. annulata* infected cells stained with a fluorescent dye. These stained cells were then examined by making smears of the afferent lymph on a cytocentrifuge. *T. annulata* infected cell lines were isolated from the afferent lymph cells (ALC) and were BoLA typed.

## **6.3 MATERIAL AND METHODS**

### **6.3.1 Animals**

Ten calves aged 2-8 months were used in these experiments. They were BoLA typed by the microlymphocytotoxicity test (Spooner *et al*, 1979) as described in chapter 3

to ensure that BoLA types of the immunised animals were different from the immunising cell line (*T. annulata* (H) 10769). The left and right prescapular lymph nodes of all these calves were removed at the age of 2-3 months as described in chapter 3. A total of 20 pseudoafferent lymphatic ducts were cannulated as described below.

### **6.3.2 Cannulation of pseudoafferent lymph vessel**

Prescapular lymph nodes were removed before the cannulations. Pseudoafferent lymph vessels were cannulated with different types of polyvinyl and polyurethane single and double lumen cannulae as described below:

#### **6.3.2.1 Cannulae for lymphatic cannulation**

Different types of cannulae were tried for this experiment as detailed below:

1) Medical grade polyvinyl single lumen tubes with an outer diameter of 1.25mm, 1.50mm or 1.75mm and double lumen tubes (Critchly Electrical Products Pvt. Ltd. Australia) with an outer diameter of 1.75 and an inner diameter of 0.70mm were used for pseudoafferent cannulation. The cannulae were impregnated with heparin before use (Nichani, 1995). Briefly, they were rinsed with acetone (BDH), filled with 2% solution of 3-aminopropyltriethoxy silane (Sigma) in acetone and left for about 5 min. After a second rinse with acetone, the cannulae were finally filled with heparin (5,000 iu./ml, Leo Lab. Ltd.) and knotted at both ends. They were wrapped in aluminium foil and stored at 4°C.

2) Special hydromer coated polyurethane catheters (Access Technologies, USA) with outer diameters of 1.1 or 1.75mm were also tried for cannulating the afferent duct. This highly sensitive catheter has according to the manufacturers, a hydromer coating

which is activated upon contact with blood/lymph to provide a highly lubricious surface for the easy flow of blood / lymph.

#### **6.3.2.2 *Preoperative procedures and anaesthesia***

The animals were starved for a day before anaesthesia. The anaesthesia was induced by the intravenous injection of 10% thiopentone sodium (RMB Animal Health Ltd.) at 1ml/10kg body weight and intubated immediately using a cuffed endotracheal tube. Anaesthesia was maintained by a mixture of halothane, nitrous oxide and oxygen using a closed circuit anaesthetic machine (BOC Inc. USA) fitted with a CO<sub>2</sub> absorber and rebreathing bag. Arterial oxygen saturation (SaO<sub>2</sub>) and tidal carbon dioxide (ETCO<sub>2</sub>) were monitored using a monitor (4700 Oxicap, BOC Inc. USA). A large area around the site of prescapular lymph node was clipped and scrubbed. Two ml of blue dye (Bleu patent V, Laboratories Guerbet, Cedex) was injected subcutaneously (s/c) at 4 different sites above and around the original site of prescapular node for easy identification of the pseudoafferent vessel draining the immunising site. The injection of dye was not required for the removal of lymph nodes. The site was sprayed with a 5% hibitane (ICI) to disinfect the area.

#### **6.3.2.3 *Operative procedure for cannulation of pseudoafferent vessel***

Both prescapular lymph nodes were removed (as described in chapter 3.3.6) and calves left free for a minimum of 8 weeks to allow the afferent lymphatics to anastomose with the efferent duct. Cannulation was performed after a minimum of 8 weeks of lymph node removal.

An incision was made about 2.5cm above and parallel to the jugular vein directly below the actual site of the prescapular lymph node which had been removed 8 weeks

before. A thin layer of brachiocephalicus muscle was divided by blunt dissection and held open with the help of self-retaining retractors. The area under the muscle was exposed. The efferent lymphatics (pseudoafferent) follow the anterior surface of fat surrounding the artery and vein. The pseudoafferent duct was identified by the blue dye which usually drained to the duct within 20-30min. after its injection (Fig. 6.1). Smaller accessory ducts were sometimes present and these were tied off before cannulating the main duct. The pseudoafferent duct was exposed without separating it from the supporting fat. A braided silk tie was made at the lower end of the exposed area of duct and pulled tight. The duct then filled up with lymph and was easier to nick. One more tie was made about 2 cms above the first tie and the first throw was made but not pulled tight. The heparin impregnated tube immersed in alcohol was rinsed in PBS and passed subcutaneously via a nick in the skin from about 5cm anterior to the main incision site using a disposable catheter passer (Codman). The proximal end of tube was cut at 45° and positioned for inserting it into the duct. The duct was nicked using spring scissors (size 15cm, Interfocus Ltd.) and a lachrymal probe (Arnolds Vet Products) of small size was inserted towards the proximal end of duct. Valves were usually encountered; these were broken with the probe to provide at least 1cm of free duct above the top tie. The cannula was inserted slowly and carefully into the duct and tied in. Blue colour lymph came out through the double lumen cannula (Fig. 6.2). The lymph flow was checked by lifting the distal end of the tube. A few cms of free tube was also tied in the area before closing the wound. The wound was closed as mentioned in chapter 3. The cannula was secured with the help of a purse string suture, at the point where it left the skin.

#### **6.3.2.4 *Post surgical procedure and care***

A plastic collar was applied around the neck of the animal. A plastic single day

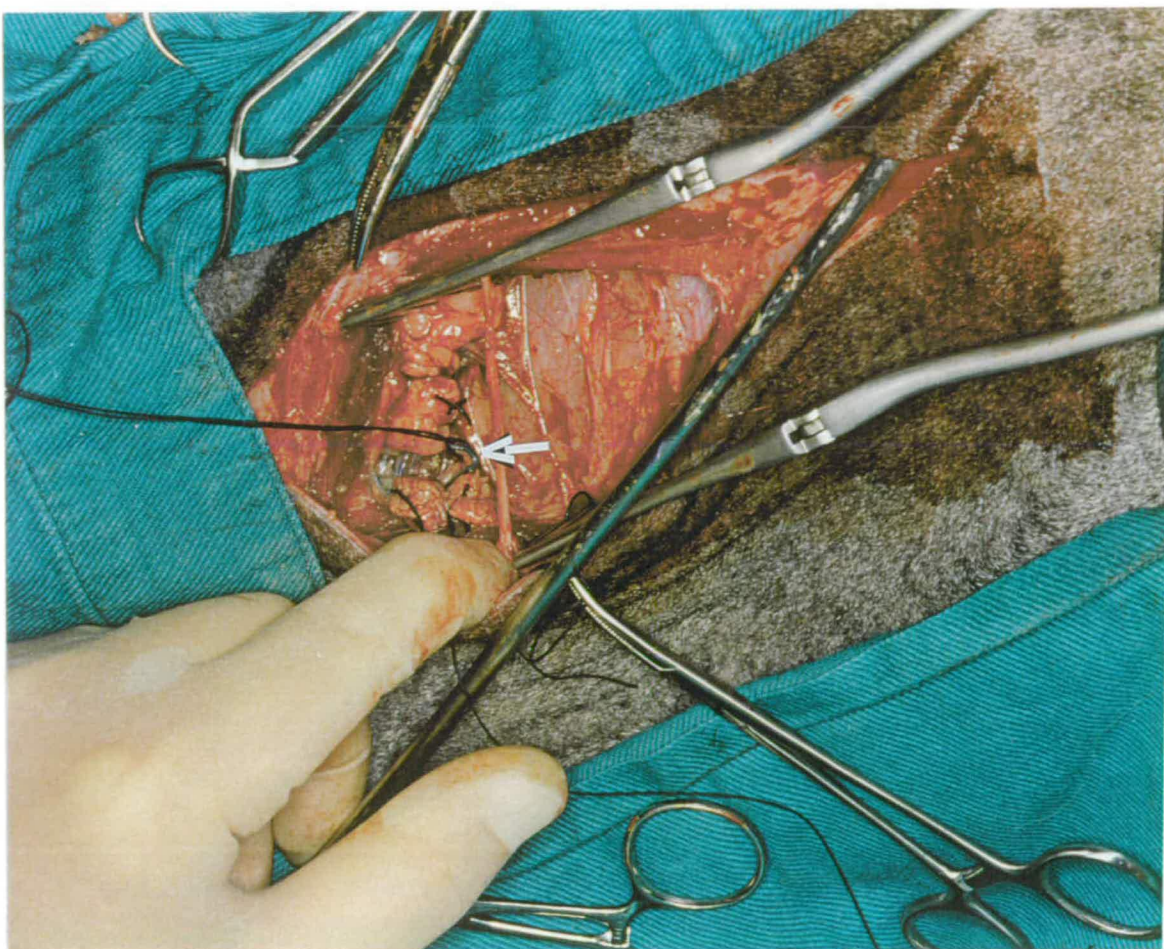


Fig. 6.1 Pseudoafferent duct (†) can be identified by the presence of blue dye.



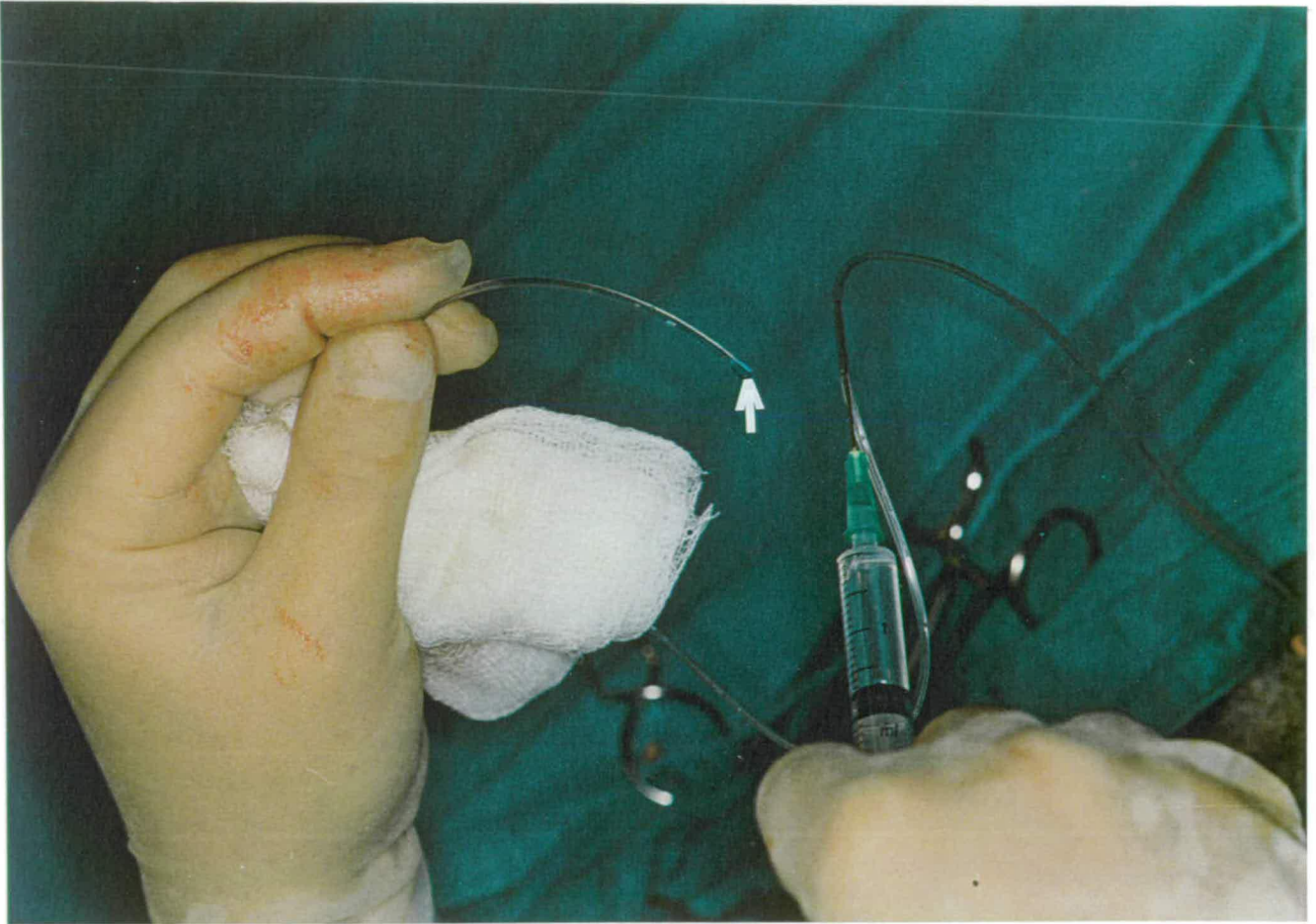
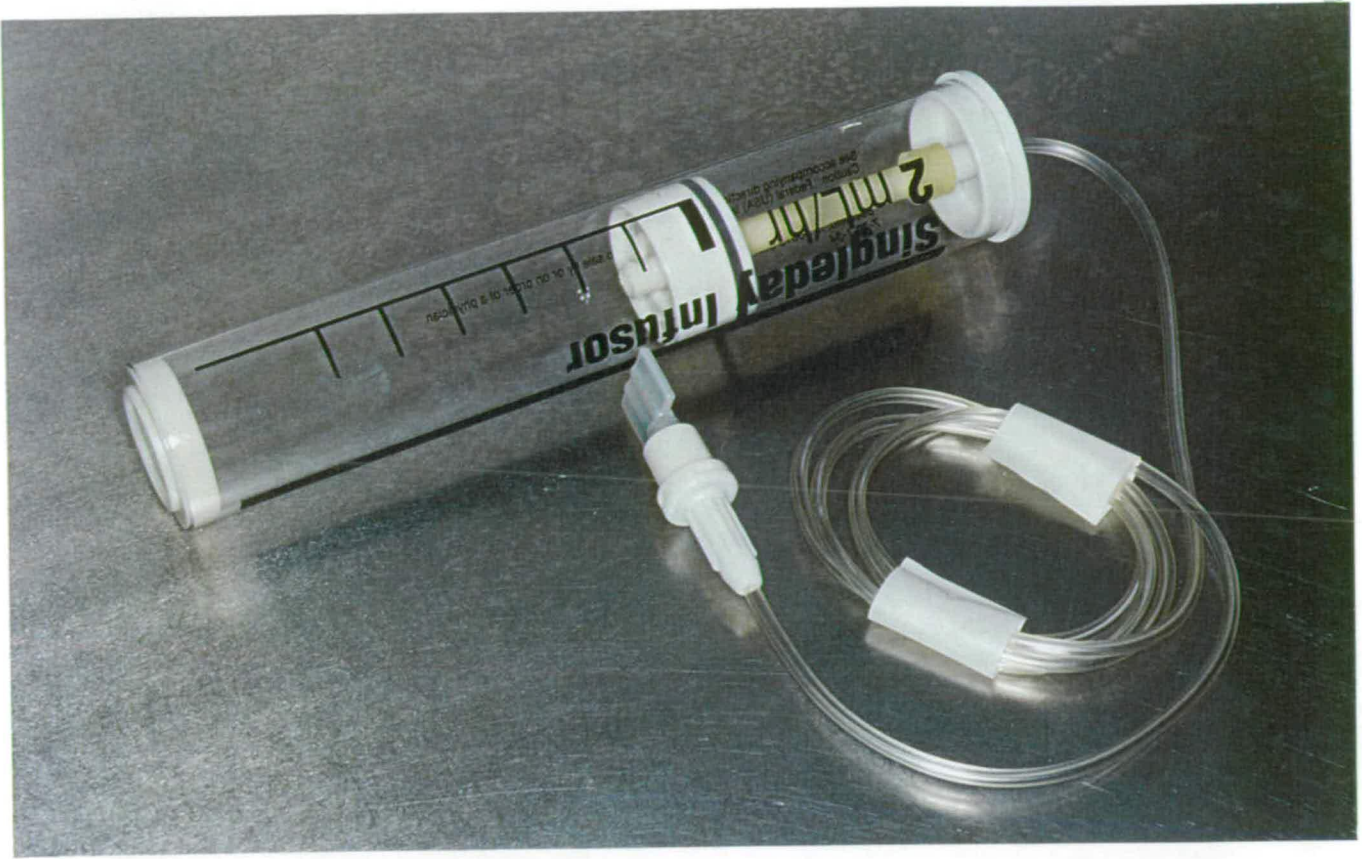


Fig. 6.2 Blue colour lymph (↑) draining from the double lumen cannula.

infusor (Baxter, UK, Fig. 6.3) was filled with anticoagulant and attached in the upright position to the collar with the lid facing the top. The tube coming out of the distal end of infusor was attached to a blunt 21 gauge(#) needle which was inserted very carefully in to one lumen of the double lumen cannula which had previously been punctured with a sharp 21# needle (Fig. 6.4). The infusor and the needle were secured very carefully and anchored with medical grade adhesive tape. A sterile plastic bottle was attached to the collar below the distal end of the infusor. The free end of the cannula was placed inside the bottle for collection of lymph. The concentration of anticoagulant (heparin) was calculated based on the flow rate of the lymph. 10 iu were required for 1ml of the lymph. Half of it was put in the collecting bottle and the other half was supplied through the infusor. The concentration of heparin in the infusor was adjusted over the subsequent days depending on the flow rate of saline/ hour through it. The infusor was supposed to supply about 2ml/hr, but actually did not supply more than 1.5ml/hr. The remaining heparin and antibiotics, to provide penicillin 20 iu and streptomycin 20µg/ml by the volume, were added to the bottle. The collar, infusor, cannula and bottle were secured by covering them with an expanding netting as used for supporting limbs in human medicine (Surgifix, FRA, Italy)(Fig. 6.5). Then animal was removed to the recovery pen. Normally animals were on their feet within 2 hrs of the operation.

Lymph collected before immunisation was reinfused slowly into the animal through a jugular cannula to minimise the loss of fluid and cells and to maintain the fluid, electrolyte and protein balance.

a)



b)

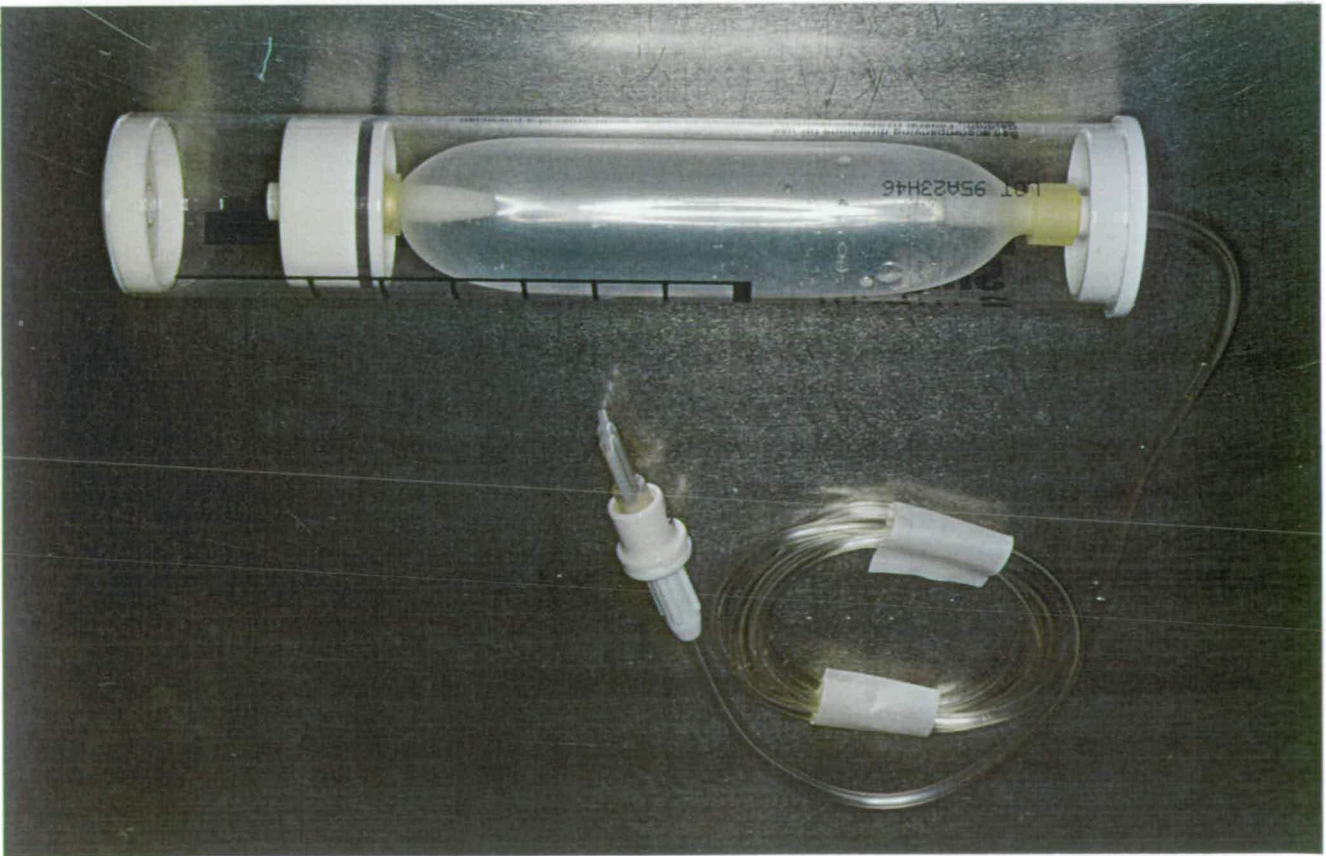


Fig. 6.3 Single day infusor. a) empty balloon; b) balloon filled with anticoagulant and saline.





Fig. 6.4 Distal end of the tube (1) attached to a needle (green, 1).

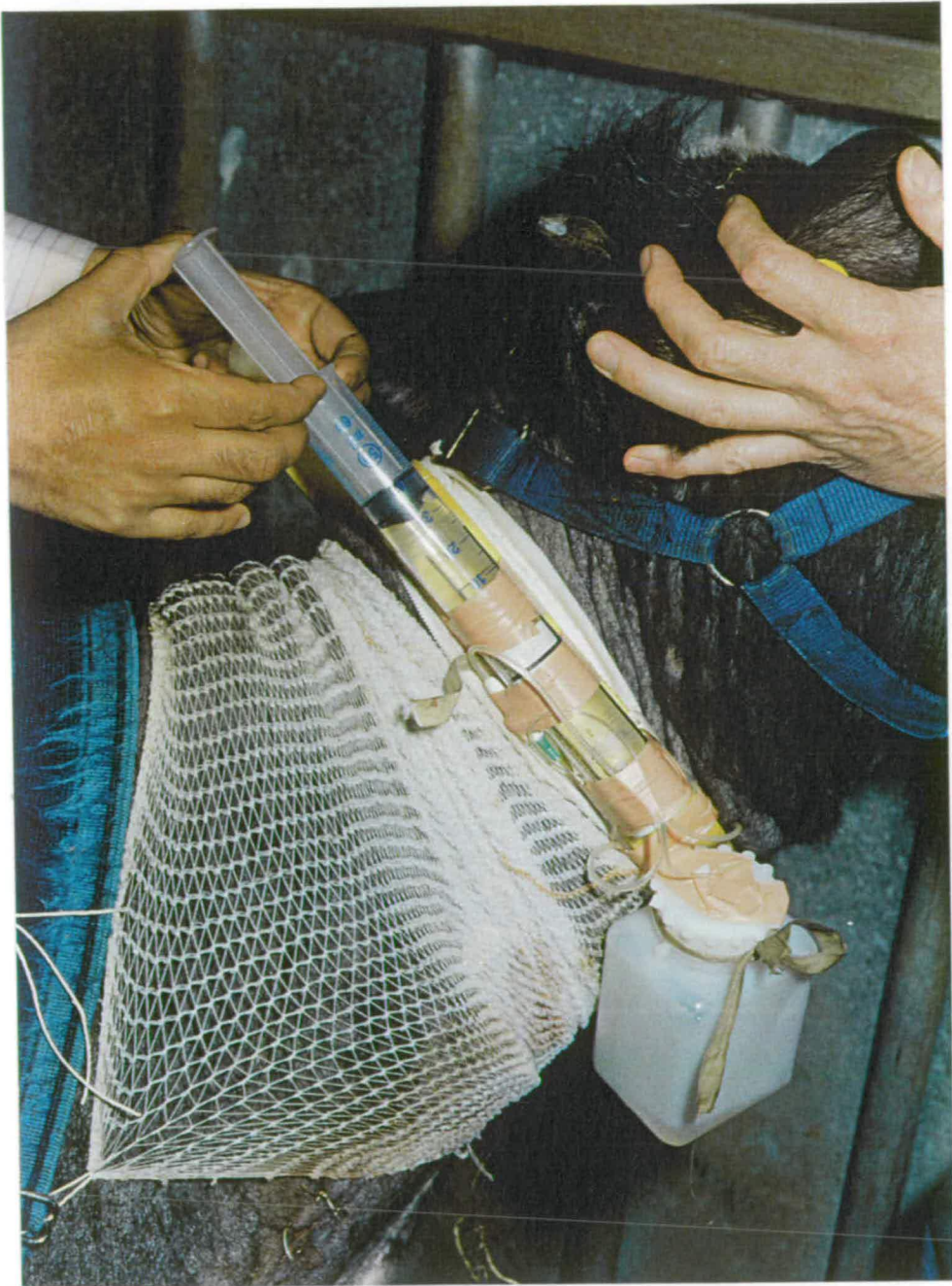


Fig. 6.5 Bottle, infusor and netting on the calf showing how the infusor could be filled easily with a 50ml syringe.

### **6.3.3 Immunisation of cannulated animals with a *T. annulata* cell line stained with a fluorescent dye.**

The cannulated animals were immunised s/c, 5 days after the surgery above the prescapular lymph node with  $10^6$  cells of *T. annulata* (Hissar) immunising cell line stained with a fluorescent dye.

#### **6.3.3.1 Cell lines**

Macroschizont-infected cell lines were established *in vitro* by infection of PBM with *T. annulata* sporozoites as described previously (Brown, 1983) and detailed in chapter 3(3.3.1). The *T. annulata* (H) 10769 cell line was used to immunise animals 5 days after cannulation. The immunising cell line was stained with a fluorescent dye (PKH2, Sigma).

#### **6.3.3.2 Staining of a cell line with a fluorescent dye (PKH2)**

*T. annulata* infected cells in their exponential phase were used for staining.  $2 \times 10^7$  *T. annulata* infected cells were washed in RPMI by centrifuging at 200g for 5 min. Supernate was removed and the cell pellet resuspended with the remaining RPMI in the tube. The cells were diluted in 1ml of diluent provided in the kit (PKH2, Sigma) and mixed gently. The dye was diluted immediately before use by mixing 5 $\mu$ l of stock solution with 1ml of diluent. The cells were then added to the 1ml dye in a separate tube, mixed gently by pipetting and incubated for 5 min. at room temp. with a further mixing during incubation. Cells were incubated for 1 minute with an equal volume (i.e. 2ml) 100% serum to stop the reaction. After the addition of a further 4ml of serum, the cells were centrifuged, transferred to a separate tube and washed thrice with TC. The staining of cells was examined on cytopsin smears under fluorescent microscopy and the cell concentration was adjusted to  $10^6$  /ml before

immunisation.

The decay of the dye was examined in a pilot experiment using stained cells maintained in TC medium. Stained cells were easily identifiable by flow cytometry and under fluorescent microscope up to 5 days with a gradual decay up to day 10. No toxic effect of dye and diluent on the growth of *T. annulata* infected cells in culture medium was observed.

#### **6.3.4 Collection of lymph and preparation of afferent cells after immunisation**

The bottle was first changed within half an hour after immunisation and thereafter every hour up to 5-6 hours on the day of immunisation. The bottle was then changed twice a day depending upon the flow of lymph. The volume and cellularity of the lymph was measured after every collection to calculate rate of lymph flow and cellularity. Cells collected during the first half an hour and then every hour after immunisation, on the day of immunisation, were counted in a haemocytometer and observed under fluorescence microscope for the presence of dye labelled cells of immunising cell line. Cells collected overnight were used for flow cytometry, proliferation assays and cytotoxicity assays (see next chapter). An aliquot of lymph was centrifuged at 800g for 15 minute and cell-free lymph plasma supernate was decanted and stored at -20°C for IFN $\gamma$  estimation. ALC were washed twice in RPMI for any assays. ALC and PBM were cultured *in vitro* in 24 well plates (Intermed, Nunc) to isolate parasite infected cell lines for studying the kinetics of dissemination of parasite infected cells. Cultures were established daily from ALC and on alternate days from PBM.

### **6.3.5 Clinical monitoring**

The clinical condition of the animals was assessed as described in chapter 3. Cytospin smears from afferent lymph were prepared daily and stained with Giemsa for assessment of blasting cells and presence of *T. annulata* infected schizonts. Blasting cells were also assessed by FACS analysis using forward scatter (FSC) vs side scatter (SSC) to identify large cells. Percentage of piroplasm infected erythrocytes was assessed by thin blood smears stained with Giemsa stain.

## **6.4 RESULTS**

### **6.4.1 Cannulations**

Cannulations were started in February, 1994. Initially both single & double lumen cannulae were used with the animal restrained and the pump and collecting bottle separate from the animal. Such prolonged restraint of the calves was thought to be unsatisfactory, due to the possibilities of the stress induced by restraint, affecting the results. A first attempt to avoid restraint involved trying a new specialised hydromer coated catheter, unfortunately the cells clotted within 2 days. In 1995, the idea of attaching the light weight infusor to the animal was tried and proved highly successful.

A total of 16 pseudoafferent ducts were successfully cannulated. Of these 3 were cannulated with an anticoagulant treated single lumen cannula, 6 with a single lumen hydromer coated polyurethane catheter, 2 with a double lumen cannula attached to a large heavy peristaltic pump kept away from the animal and 5 with a double lumen cannula attached to a light-weight plastic single day infusor attached around the neck of the animals. Although 16 were cannulated only 3 cannulae ran for long enough to monitor the ALC. Thus out of the last 7 cannulations, 3 ran for 4 days, 1 for 5 days



and 3 for 15-20 days (13054, 12886, 13130). Where an infusor was attached to the calves, the calves were free to move around in their pens with easy access to food and water (Fig. 6.6 & 6.7).

#### **6.4.2 Clinical reactions**

##### **6.4.2.1 *Temperature***

All three animals (13054, 12886, 13130) showed febrile reactions in the form of a rise in temperature after immunisation (Fig. 6.8a). The first peak of temp. was noticed during the first 8 days of immunisation. Calf 13054 showed a peak temp. of 40.7°C on day 6, while 12886 and 13130 showed peaks of 40.7°C & 41.1°C on days 8 and 6 respectively. The second peak of temperature occurred during the 3rd week of immunisation when it was 40°C and 40.3°C on day 18 in animals 12886 and 13130 respectively. No second peak was observed in calf 13054.

##### **6.4.2.2 *Haematological observations***

A decrease in TLC, TEC and PCV was observed in all the three animals. The decrease in PCV was more pronounced in the later stages of immunisation.

##### **6.4.2.3 *Parasitological observations***

Mild parasitological reactions were observed with the appearance of piroplasms in the peripheral blood after cell line immunization (Fig. 6.8b). The piroplasms were first detected on days 11, 16 and 13 in the peripheral blood of 13054, 12886 and 13130 respectively. A maximum of 3-4% piroplasms were detected in animals 13054, 12886 and 13130. As the draining lymph nodes were removed in all the animals, lymph node biopsies could not be made to detect schizonts.



Fig. 6.6 A flask full of blue lymph collected immediately after cannulation.



Fig. 6.7 Calf is free to move around with infusor and bottle attached to its neck and covered by netting.

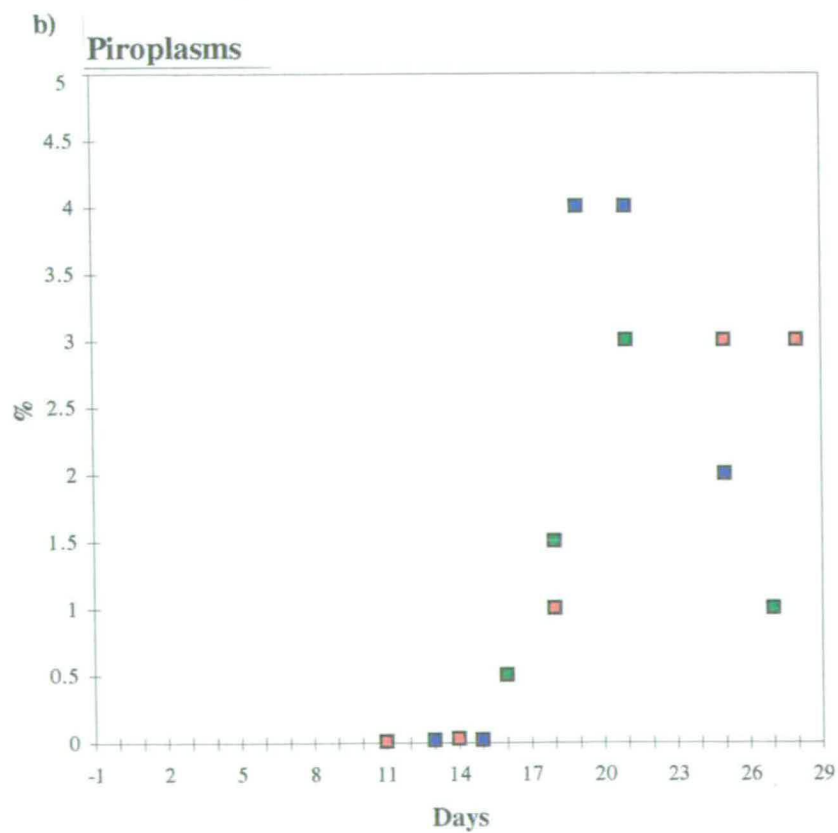
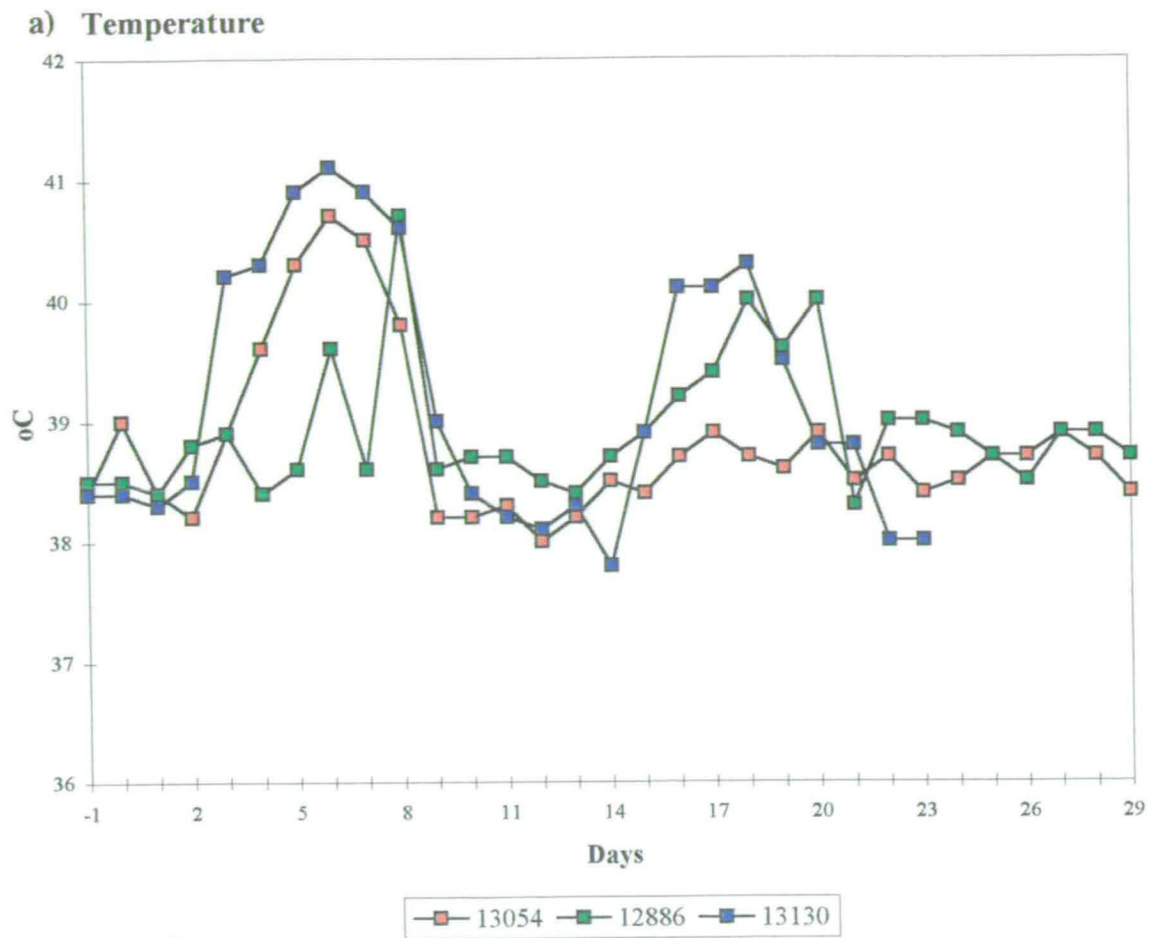


Fig. 6.8 a) Temperature and b) piroplasms of calves 13054, 12886 and 13130 following immunisation with *T. annulata* (10769) cell line.



#### **6.4.3 Changes in the lymph flow and cellularity**

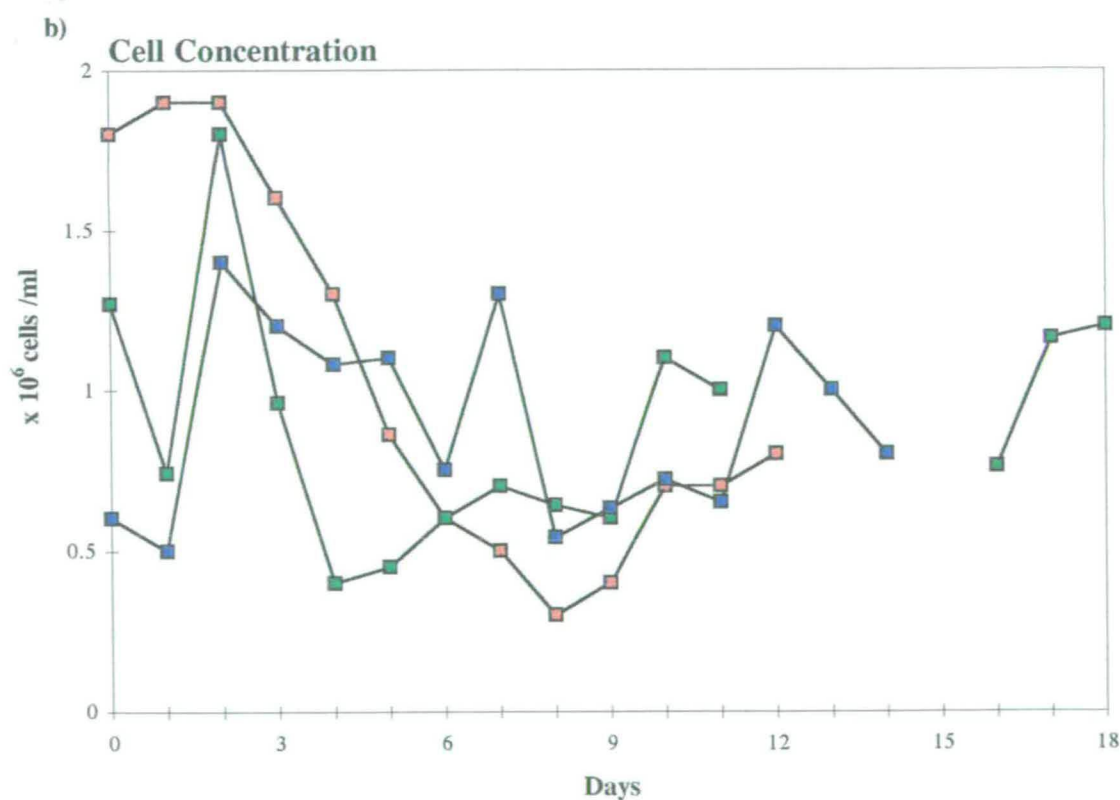
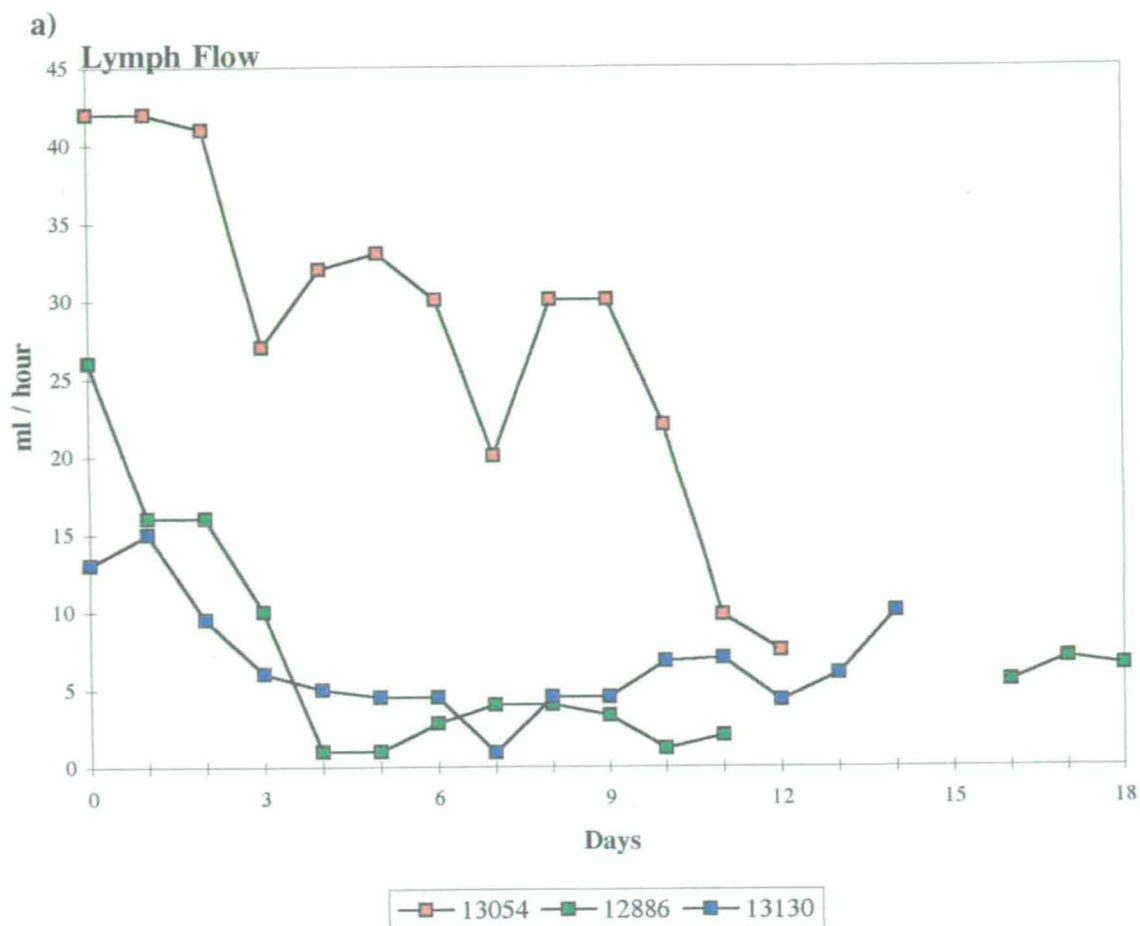
The flow of afferent lymph draining the site of immunisation decreased in all the animals (Fig 6.9a) with a sharp decline around days 6 and 7 after immunisation. The flow rate never returned to the normal level in any of the three animals during the observation period. Output of cells in the afferent lymph decreased sharply in animals 13054 and 12886 but there was little change in animal 13130 (Fig. 6.9b). Cell numbers returned back to their base levels in animals 12886, 13130 but not in 13054.

#### **6.4.4 Kinetics of parasite dissemination / parasite transfer**

Fluorescent dye stained cells of the *T. annulata* infected immunising cell line were observed in the afferent lymph in all the three animals (Fig. 6.10) within half an hour after immunisation. As monitored by microscopy the concentration of cells increased during the first hour in animal 12886 but during the third hour in animal 13054. Few cells were observed in animal 13130 as compared to other two animals.

*T. annulata* infected cell lines were isolated from the afferent lymph during the first 24 hours after immunisation from all the three animals. All the three isolated cell lines were BoLA typed and were found to be of the donor cell line origin.

Piroplasms were detected in all three animals after day 11. Schizont infected cells were isolated from PBM of animal 12886 from day 21 onwards, animal 13130 from day 16 onwards and were shown to be of recipient origin by BoLA typing. No schizont infected cells were isolated from animal 13054 even after intensive sampling up to 1 month and again after 4 months of immunisation.



**Fig. 6.9 a) Lymph flow and b) cell concentration in the afferent lymph of calves 13054, 12886 and 13130 following immunisation with *T. annulata* (10769) cell line.**  
**Day 0 represents lymph collected before immunisation.**

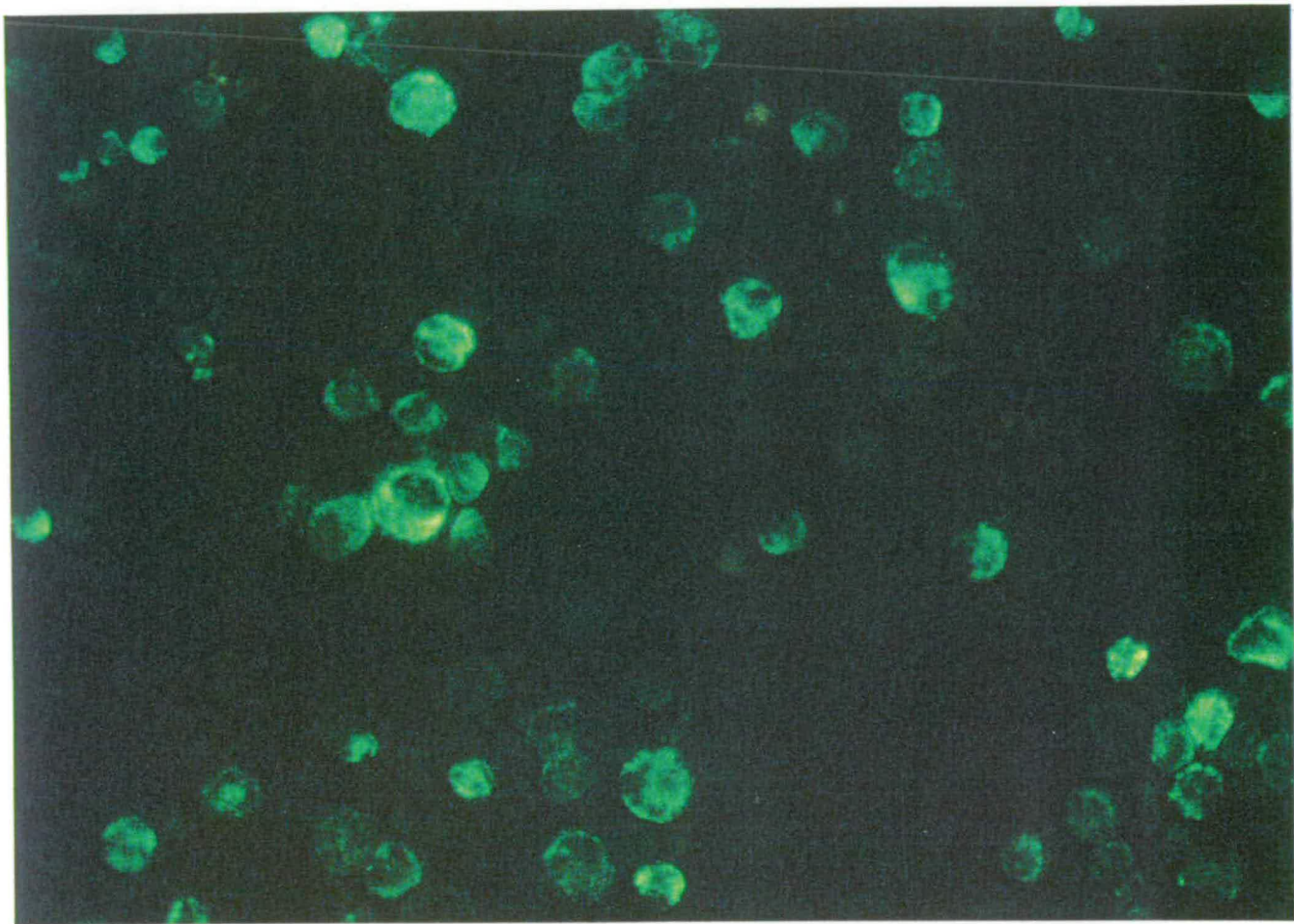


Fig. 6.10 Fluroscent dye stained cells (green) collected from the pseudoafferent duct after immunisation.

## 6.5 DISCUSSION

The cannula with a double lumen medical grade polyvinyl tube attached to a plastic infusor was highly successful. This was the first time that cannulation of bovine lymphatics had been maintained in unrestrained and hopefully less stressed animals.

A decrease in the flow rate of afferent lymph and total cell output was observed in all the animals after immunisation. A similar "cell shutdown" in the draining lymph node after antigen challenge (Hall & Morris, 1965b; Hopkins *et al*, 1981) and after sporozoite challenge (Nichani, 1995) has been reported previously. It is possible that cell shut down in the draining node and efferent lymphatics (Nichani, 1995) had actually taken place in the skin at the site of challenge. In contrast, an increase in flow rate and cell output was observed in the efferent lymph after cell line immunisation (Nichani, 1995), after inoculation of allogeneic lymphocytes in the draining area (Emery & McCullagh, 1980) and after *Toxoplasma* infection in sheep (Innes *et al*, 1995). This might have resulted from an increased blood supply and recirculation of naive lymphocytes in response to allogeneic cells (Hay & Hobbs, 1977). The cell shut down phenomenon described here may have indicated a reduction in the draining capacity of the afferent lymph after cell line immunisation due to the retention of cells at the site of immunisation or a reduction in the flow rate of lymph from interstitial tissues to the collecting afferent lymph vessels.

This study has shown for the first time that *T. annulata* infected cells of the immunising cell line drained immediately to the draining lymph node after immunisation. In all three animals, PKH2 stained cells of the immunising cell line drained into the collecting bottle within minutes of injection. The *T. annulata* infected cell lines reisolated from the stained cells taken from the afferent lymph immediately



after cannulation were of donor origin as confirmed by BoLA typing. The draining lymph node was presumably therefore the site of transfer of parasites from the donor cells to the cells of the recipient calves, suggesting the afferent lymphatics were the main route of parasite dissemination from the site of immunisation to the draining lymph node. Since all the cells draining through the afferent lymphatic system were collected the presence of the parasite in the blood of all 3 animals indicated that some of the cells followed another route after immunisation. In the present experiment infected donor cells appeared in the afferent lymph within minutes of immunisation. However a previous study showed that no cells of donor origin occurred in the efferent lymph and that the first infected cells of recipient origin were only seen on day 12 (Nichani, 1995). Taken together, these observations suggest that the infected donor cells are residing in the lymph node draining the site of immunisation, up to day 12 and it seems highly likely that the parasite transfers from the donor to the recipients cells in the node during this time. In the present experiment, it was only possible to reisolate cell lines from PBM in two of the three animals and in both the cases the cell lines were of recipient origin. However, piroplasms developed in all 3 animals and all were immune when challenged with a lethal dose of heterologous *T. annulata* (Gharb) sporozoites (chapter 7). These observations indicated that cells might reach the circulation by routes other than the prescapular node.

To summarise, a novel method of cannulation was developed successfully. The presence in the afferent lymph of both fluorescent inoculated cells and infected cells expressing the donors BoLA type indicated that the parasites transferred from donor to recipient cells in the DLN. This method of cannulation was then applied to studies on T cell activation in the pseudoafferent lymph of calves immunised with *T.*

*annulata* infected cell lines as described in the next chapter.

**CHAPTER 7**  
**T CELL ACTIVATION IN THE AFFERENT LYMPH OF CATTLE**  
**FOLLOWING IMMUNISATION WITH *THEILERIA ANNULATA***  
**INFECTED CELL LINES.**

## 7.1 INTRODUCTION

Previous studies showed T cell activation in the DLN (chapter 3,4 & 5), in PBM (Innes *et al*, 1989a) and in the efferent lymph of cattle (Nichani, 1995). Both a transient appearance of MHC class I restricted cytotoxicity (Innes *et al*, 1989a) and a more sustained macrophage mediated cytotoxic activity against parasite infected cells (Preston & Brown, 1988) have been demonstrated after immunisation with *T. annulata* infected cell lines. A transient increase in circulating CD8<sup>+</sup> cells and a sustained presence of monocytes/macrophages in the peripheral blood have been reported after cell line immunisation and the progression of the disease was reported to be associated with a non-specific activation of T cells with the loss of CD2 on the CD8<sup>+</sup> T cells alongwith extremely high levels of IFN $\gamma$  in the efferent lymph (Nichani, 1995) and DLN (Campbell, 1995). IFN $\gamma$  production in the efferent lymph was 20 times less in immunised animals than in susceptible animals infected with lethal dose of sporozoites (Nichani, 1995). In a recent study, activated CD4 and CD8 cells left the node draining the site of immunisation with an allogeneic *T. annulata* infected cell line in the efferent lymph. Functional assays showed these cells to be specific for allogeneic antigens as well as parasitised autologous cells (Nichani, 1995). Similar findings were observed in the DLN (chapter 3 & 4 of this thesis). In reimmunisation studies with a *T. annulata* infected cell line, pre-existing allogeneic responses blocked the parasite specific immune responses in some animals (Nichani, 1995). This finding indicated that the allogeneic response stimulated after primary immunisation might have killed the cell line used for reimmunisation at the site of inoculation.

Bovine afferent lymph contains about 50% T cells (Emery *et al*, 1987). However, the level of activation and immunocompetence of these T cells is unknown and their involvement in the systemic dissemination of the immune responses is unclear. To

date no study has been undertaken to understand the activation of T cells and immune responses at the site of immunisation with cell lines. The site of immunisation is of interest as the first site of initial cellular interaction between T cells, APC and the cells of the immunising cell lines. Since pseudoafferent cannulation is the only way to study T cell activation at the site of immunisation, this method was used on cattle immunised with cell lines with the following objectives:

- i) To study the dynamics of various lymphocyte subpopulations and NK cells and the activation of T cells at the site of immunisation.
- ii) To examine the immune status of the immunised calves, which lack the DLN.

## **7.2 EXPERIMENTAL DESIGN**

Afferent lymph cells (ALC) were collected by cannulating the afferent duct. Animals were immunised and the ALC were analysed for their subpopulation and activation by flow cytometry. Preliminary investigations of natural killer like cells (NK) was undertaken by flow cytometric analysis. Functional specificity of ALC was assessed by *in vitro* proliferation and cytotoxicity assays. Immune status of the immunised animals (13054, 12886) was assessed by challenge with a lethal dose of sporozoites.

## **7.3 MATERIALS AND METHODS**

Afferent lymph was collected and the cells were separated for analysis as described in chapter 6 (6.3.4). Flow cytometric analysis and functional assays were performed as detailed below.

### **7.3.1 Analysis of afferent cells using flow cytometry**

Flow cytometry is an analytical technique which permits accurate quantification of various parameters of individual cells (size, complexity and fluorescence) in a

population of continuously flowing fluorochrome labelled cells. In this study, flow cytometric analysis was performed with a FACScan flowcytometer (Becton Dickinson, USA). This equipment has a single argon laser which produces a beam of 488nm wavelength. As the cell passes through the focused laser beam, it scatters laser light and the fluorescent molecules on its surface are excited to fluoresce. A forward light scatter (FSC) is directly proportional to size of the cell. The signals collected at 90° give information on cell complexity and are termed "side scatter" (SSC). Fluorochrome bound to the cell surface absorbs the laser light and emits a light of longer wavelength. FITC emits light at 530nm and phycoerythrin (PE) at 580nm. Fluorescent light is collected by a lens and the different colours produced by different dyes are directed to separate photomultiplier (PMT) detectors. Data on these parameters can be stored and analysed in a computer.

#### *7.3.1.1 Bovine leucocyte specific monoclonal antibodies*

Monoclonal antibodies which react with bovine leucocyte sub-populations were used in this study. The specificities of these antibodies have been tested in two international workshops on bovine leucocyte antigens (Howard *et al*, 1991a; Howard and Naessens, 1993). A summary of the mAbs and the molecule they detect is given in Table 7.1

The mAb IL-A19 is specific for MHC class I antigens on the cell surface (Bensaid *et al*, 1989). This mAb was used as a positive control in all the tests as all the viable cells should express MHC I on their cell surface. IL-A12 and CC8 identify a distinct subpopulation of T lymphocytes, bearing the bovine CD4 molecule (Baldwin *et al*, 1986; Teale *et al*, 1986; Howard *et al*, 1991b). SBU-T8 (Maddox *et al*, 1986) recognise bovine CD8. IL-A26 is specific for bovine CD2 (Baldwin *et al*, 1988b).

**Table 7.1**  
**Monoclonal antibodies used for flow cytometry**

mAb	Isotype	Specificity	Dilution
IL-A19	IgG <sub>2a</sub>	MHC class I	1:1000
IL-A12	IgG <sub>2a</sub>	CD4	1:1000
CC 8	IgG <sub>2a</sub>	CD4	1:1000
SBU-T8	IgG <sub>2a</sub>	CD8	1:1000
IL-A26	IgM	CD2	1:1000
MM1A	IgG <sub>1</sub>	CD3	1:100
CC 15	IgG <sub>2a</sub>	$\gamma\delta$ T cells	1:1000
IL-A111	IgG <sub>1</sub>	CD25	1:1000
CC 76	IgG <sub>1</sub>	CD 45RB	1:2000
IL-A 150	IgG	CD45RO	1:1000
IL-A30	IgG <sub>1</sub>	Surface IgM	1:1000
IL-A24	IgG <sub>1</sub>	Macrophages	1:1000
IL-A109	IgM	Monocytes	1:1000
VPM 65	IgG	CD 14	1:1000
VPM 5	IgM	CD 1	1:1000
VPM-30	IgM	Pan B cells	1:1000
J11	IgG <sub>1</sub>	MHC class II	1:1000
IL-A21	IgG <sub>2a</sub>	MHC class II	1:1500

**Table 7.2**  
**Secondary immunoconjugates**

Antibody	Conjugate	Specificity	Dilution
Rabbit anti-mouse Ig (Nordic)	FITC	Polyvalent	1:100
Goat anti-mouse Ig (Sigma)	FITC	IgM	1:100
Goat anti-mouse Ig (Sigma)	PE	IgG	1:100
Goat anti-mouse Ig (Seralab)	FITC	IgG <sub>1</sub>	1:200
Goat anti-mouse Ig (Seralab)	PE	IgG <sub>2a</sub>	1:250

FITC= Fluorescence isothiocyanate, PE= Phycoerythrin

CD2 is also expressed upon bovine CD3<sup>+</sup> cells, these are thought to represent bovine NK cells (Evans & Jaso-Friedmann, 1993). MM1A recognises bovine CD3 (Davis *et al*, 1993). WC1<sup>+</sup>  $\gamma\delta$  T cells are recognised by mAbs IL-A29 (Morrison *et al*, 1988) and CC15 (Clevers *et al*, 1990). IL-A111 is a marker for the  $\alpha$  chain of the interleukin-2 receptor (IL-2R $\alpha$ ) also known as bovine CD25 (Tac antigen) on the cell surface (Naessens *et al*, 1992). CC76 binds to the CD45RB molecule on the cell surface (Howard *et al*, 1991b). The bovine equivalent of CD45RO is recognised by IL-A150 (Bembridge *et al*, 1993). Bovine B cells expressing IgM on the surface are recognised by mAb IL-A30 (Naessens *et al*, 1988). VPM30 is specific for B cells (J. Hopkins, pers. comm.; Naessens & Howard, 1991). IL-A24 recognises a molecule present on bovine monocytes/macrophages associated with antigen presentation (Ellis *et al*, 1988). IL-A109 is thought to recognise an antigen analogous to CD64 (FcR1) on monocytes and macrophages (MacHugh *et al*, 1990). The bovine equivalent of CD14 is recognised by VPM65 (Vipin Gupta per. com.). J11 and IL-A21 recognise a monomorphic determinant on bovine MHC class II molecules (Baldwin *et al*, 1988b). All mAbs were titrated for optimal working dilutions before use.

#### **7.3.1.2 Secondary Immunoconjugates**

The immunoconjugates used in this study are given in Table 7.2. All conjugates were titrated before use to assess their optimal working dilution.

#### **7.3.1.3 Immunofluorescence staining of cells for FACS analysis**

An indirect immunofluorescence test was used to stain cells for analysis on the FACScan essentially as described by Spooner *et al*. (1988) and Glass & Spooner (1989).



#### **7.3.1.3.1 *Single parameter staining***

50µl cell suspension ( $5 \times 10^5$  cells) were mixed with 50µl of the mAb at a predetermined optimal dilution in a 96 well round bottom plate (Nunc) and incubated for 30 min. at 4°C on ice. The cells were washed twice in "Cell Wash" buffer (Becton Dickinson) by repeated suspension and centrifugation for three min. at 100g at 4°C. The supernatant was removed, 25µl of FITC conjugated rabbit anti-mouse (RAM) Ig (whole molecule, Nordic) at a dilution of 1:100 in FACS medium was added to each well, and the test plate was incubated for 30 min. on ice at 4°C. Plates were covered with aluminium foil to keep the cells in dark. The cells were washed three times in cell wash as described above and finally resuspended in 100µl cell wash and were analysed immediately. The cells were preserved by addition of 100µl of 1% paraformaldehyde (Sigma) solution in cell wash to each well. A negative control sample was included by incubating cells in 50µl of normal mouse serum (NMS, Seralab) at 1:1000 dilution in place of mAb for the first incubation. The second incubation was with the same fluorochrome as used for staining positive cells.

#### **7.3.1.3.2 *Double parameter staining***

Cells were treated as above and incubated simultaneously with 25µl each of two mAbs of different isotypes at half their working dilution. Incubation and washing were done as explained above for single parameter staining. This was followed by simultaneous incubation with 25µl of two appropriate secondary fluorescent reagents depending upon the isotypes of primary antibodies. These were Goat anti-mouse (GAM) IgM-FITC (Sigma) / GAM IgG-PE(Sigma); GAM IgG<sub>1</sub>-FITC (Seralab) /GAM IgG<sub>2a</sub>-PE (Seralab). One conjugate was FITC for green fluorescence and the second was PE for red fluorescence.

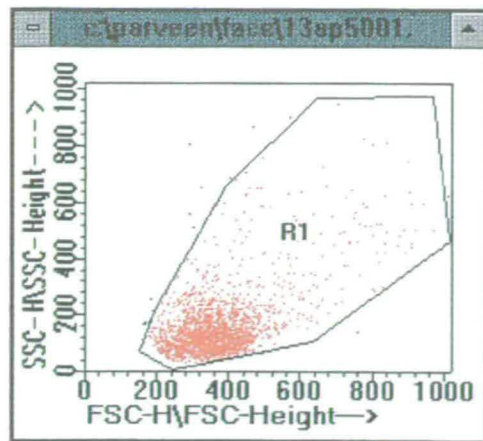
#### 7.3.1.4 FACS acquisition and analysis

The "lysyes II" programme (Becton Dickinson) was used in the acquisition and storage of both the single and two colour flow cytometric parameters of the labelled cells. The "lysyes II" and "PC lysyes" programmes (Becton Dickinson) were used for analysis of the data.

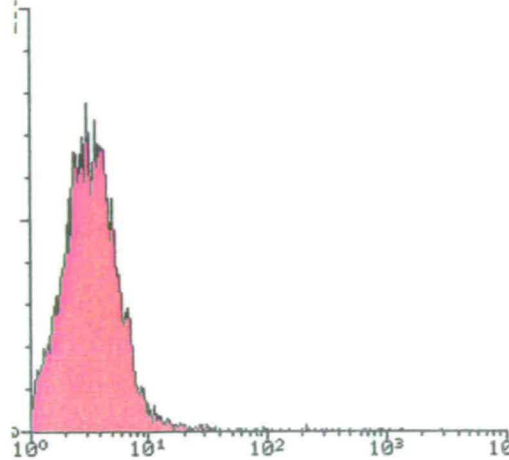
For single colour parameters, the cytometer configurations were calibrated and set using negative control cells stained with NMS and the appropriate immunoconjugates. FITC fluorescence (530nm) emission was detected with the PMT voltage set at 630-650mV on FL1 as logarithmic (log) amplification scale. FSC and SSC (90°) amplifications were linear and set at EOO (unamplified) and 400mV, respectively. Using FSC versus SSC dot plot, lymphocytes were live gated for acquisition and analysis while cell debris, dead cells, granulocytes and RBC were excluded (Fig. 7.1a). For acquisition, cell samples were run at approximately 500 cells per second (sec.) and normally data on 10,000 cells were recorded. The data was plotted either as frequency histograms with log fluorescent intensity presented on the x-axis and frequency of cell numbers presented on the y-axis (Fig. 7.1b) or as dot plots with log fluorescent intensity presented on the x-axis and FSC on the y-axis (Fig. 7.1c).

For two colour parameters, the cytometer configurations were calibrated and set as described above using the negative control cells stained with NMS and appropriate secondary conjugates. The cells were live gated by using a dot plot of FSC versus SSC. The optimum FITC (FL1) and PE (FL2) levels were adjusted to bring the cells to the lower left corner of the box (Fig. 7.2a). These optimal PMT voltages were found to be 630-650 mV and 520-540 mV for FL1 and FL2 respectively for ALC and both were in the log amplification using 1024 channels.

a)



b)



c)

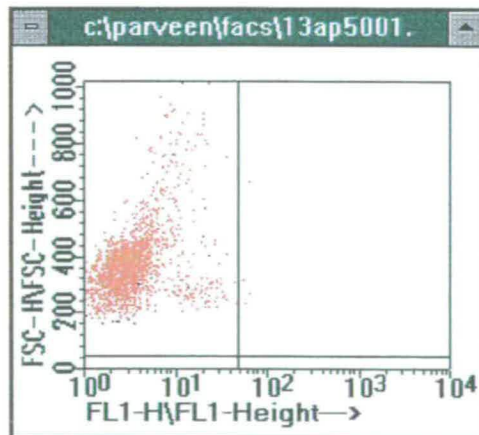


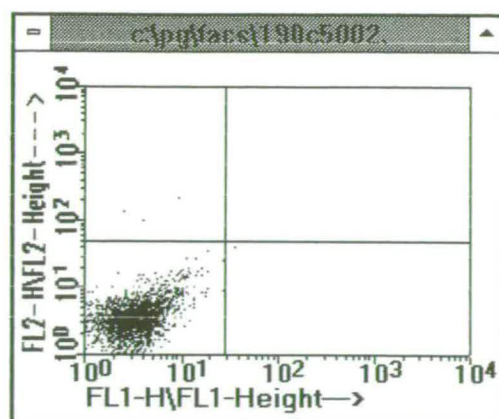
Fig. 7.1 Flow cytometric display of cells during acquisition using forward and side scatter (FSC vs SSC).

a) Dot plot display of cells (Region 1, "R1") excluding RBCs, dead cells and neutrophils.

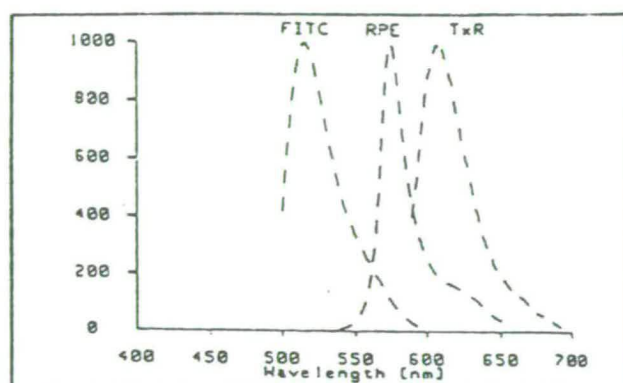
b) Frequency histogram of "R1" cells.

c) Dot plot display of "R1" cells with log fluorescence on x-axis and FSC on y-axis.

a)



b)



Fluorescence Emission Spectra of FITC, PE and Texas Red from PE-Texas Red Duochrome Conjugate only

c)

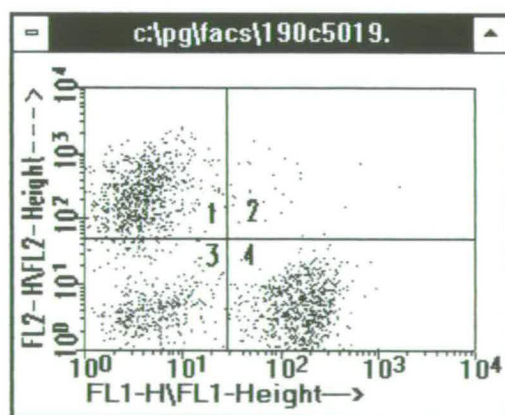


Fig. 7.2 Flow cytometric display of cells for two colour analysis.

a) Dot plot display of the negative control sample adjusted for FITC and PE voltage levels.

b) Spectral overlap from emission of FITC and PE. (From manual of FACScan, Becton Dickinson).

c) Two colour dot plot display of cells stained for CD2 antibody (FL1) and  $\gamma\delta$ T antibody (FL2). 1:  $\gamma\delta$ T<sup>+</sup> cells, 2:  $\gamma\delta$ T<sup>+</sup>+CD2<sup>+</sup> cells, 3: Double negative cells ( $\gamma\delta$ T<sup>-</sup> and CD2<sup>-</sup> cells), 4: CD2<sup>+</sup> cells.

To compensate for the unwanted spectral overlap of emitted light from the FITC and PE dyes used (fig. 7.2b), the FL1-% FL2 PMT voltage was set between 0.5-1.5% to remove PE signal from FL1 region, and the FL2-% FL1 PMT voltage was set between 30-50% to remove FITC signal from the FL2 region. These settings were done for every test with cells stained with only one primary mAb but both secondary conjugates. Sometimes these settings were slightly different for different mAbs and, therefore, changed accordingly between samples. The data was analysed using dot plots with log FL1 (FITC) intensity on the x-axis and log FL2 (PE) intensity on the y-axis. For statistical analysis, the dot plots were set into 4 calculation regions by setting quadrant markers (Fig. 7.2c). Figure 7.2c represents CD2 cells FITC (FL1) versus  $\gamma\delta$ T cells PE (FL2) plot in which quadrant 1 represents  $\gamma\delta$ T positive cells only, quadrant 2 represents  $\gamma\delta$ T & CD2 dual positive cells, quadrant 3 is the negative population for both primary antibodies while quadrant 4 is the CD2 cells.

### **7.3.2 Functional assays**

*In vitro* proliferation assays were performed as described in chapter 4(4.3.2.1) and cytotoxicity assays were performed as detailed below.

#### **7.3.2.1 Cytotoxicity Assay**

A standard 4 hour chromium release assay was used as previously described (Teale *et al*, 1985; Spooner *et al*, 1987 and Innes *et al*, 1989a).

##### **7.3.2.1.1 Effector cells**

The cytotoxic activity of ALC from the calves was assayed at regular intervals after cell line immunisation. The cells were prepared by centrifugation of afferent lymph as described above. The effector cells were finally resuspended in TC medium. The

FCS used in the medium was from a batch that had previously been screened to give low levels of spontaneous  $^{51}\text{Cr}$  release from the target cells.

#### **7.3.2.1.2 Target cells**

Both *T. annulata* infected target cells and uninfected blasts in exponential growth phase were chosen for use as target cells. The cells were washed once in TC medium by centrifugation at 100g for 10 min. and were resuspended in the same medium at a concentration of  $2 \times 10^7/\text{ml}$ . An equal volume of  $^{51}\text{Cr}$  at a concentration of 1mCi /ml in TC medium (as sodium chromate, Amersham International Ltd.) was added to each target cell suspension. The cells were incubated for 60 min. at  $37^\circ\text{C}$  and mixed gently a few times during incubation. Following incubation the labelled cells were washed three times in TC medium. After the final wash they were resuspended to give a final cell concentration of  $1 \times 10^6$  cells/ml.

#### **7.3.2.1.3 Cytotoxicity assays**

The cytotoxicity assay was performed by adding 25 $\mu\text{l}$  ( $2.5 \times 10^4$  cells) of the labelled target cell suspension to 100 $\mu\text{l}$  of effector cell suspension in 96 well round-bottom tissue culture plates (Intermed, Nunc) in duplicate. The concentration of effector cells was varied to give different effector:target ratios i.e  $1 \times 10^7$  cells per ml for a 40:1 ratio,  $5 \times 10^6$  cells per ml for 20:1 ratio,  $2.5 \times 10^6$  cells per ml for 10:1 ratio and so on. The test plates were incubated for 4 hrs in a 5%  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ . The contents of each well were individually mixed followed by centrifugation at 1000g for 5min. 25 $\mu\text{l}$  of the supernatant from each well was removed and placed in a disposable 96 well assay plate (Falcon). 100  $\mu\text{l}$  of scintillant (Optiphase safe, LKB Scintillation products) was added to each well. The plates were sealed with a plate seal (Wallac) and counted in a scintillation counter (Microbeta, Wallac). The percentage

cytotoxicity was calculated as follows:

$$\text{Specific cell lysis (\%)} = \frac{\text{Test release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

Spontaneous release of  $^{51}\text{Cr}$  from the target cells was measured by incubating 25 $\mu\text{l}$  of target cells with 100 $\mu\text{l}$  of assay medium alone and maximum release was measured by mixing 25 $\mu\text{l}$  of target cells with 100 $\mu\text{l}$  of 1% sodium dodecyl sulphate (SDS, Sigma) solution for lysis of all the cells. The spontaneous and maximum release controls were carried out in triplicate wells.

### 7.3.3 Immune status of animals

Two immunised calves (13054 & 12886) along with a naive control were challenged with a lethal dose of 1 t.e. of *T. annulata* (G) sporozoites. Their clinical and parasitological reactions were recorded.

## 7.4 RESULTS

### 7.4.1 Analysis of afferent cells using flow cytometry

#### 7.4.1.1 Kinetics of lymphocyte subpopulations in the afferent lymph

The kinetics of the various lymphocyte subpopulations in afferent lymph after immunisation is shown in Fig. 7.3 to Fig. 7.5.  $\text{CD2}^+$  cells were found to be between 22% to 30% of total cells in the resting afferent lymph of the two calves (12886, 13130) (Fig. 7.3). They started to increase by day 4, reached their peak level between day 8 to 12 and declined thereafter but never returned to the resting level as long as the cannulae kept running in both the animals (Fig. 7.3). A slight change in the level of  $\text{CD4}^+$  cells was observed with a sharp decline in one of the two animals (Fig. 7.4). The level of  $\text{CD8}^+$  cells increased dramatically in all the animals after

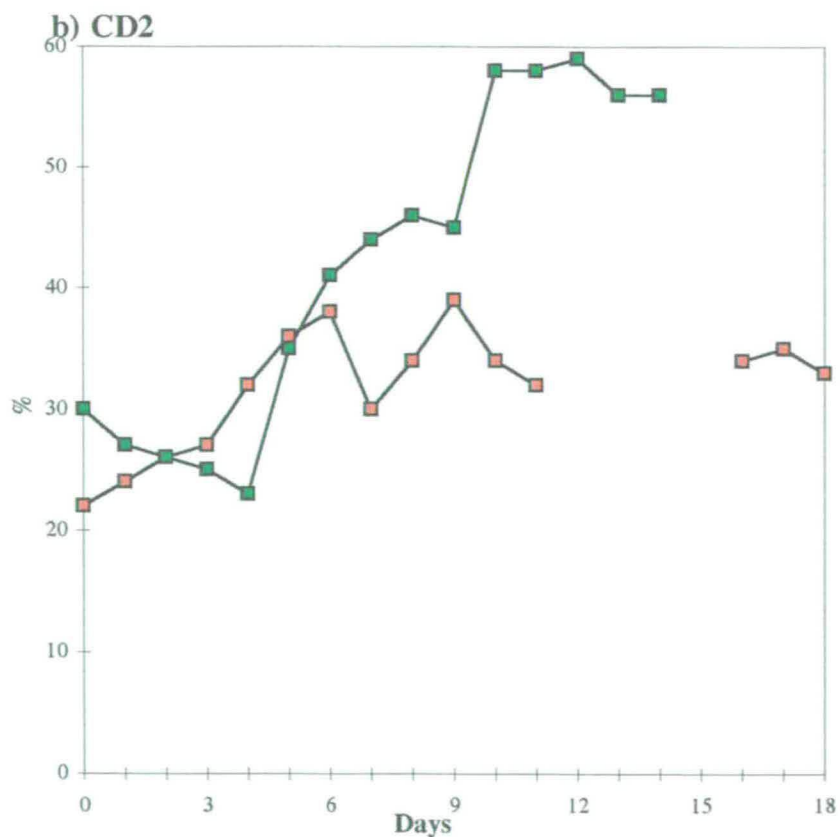
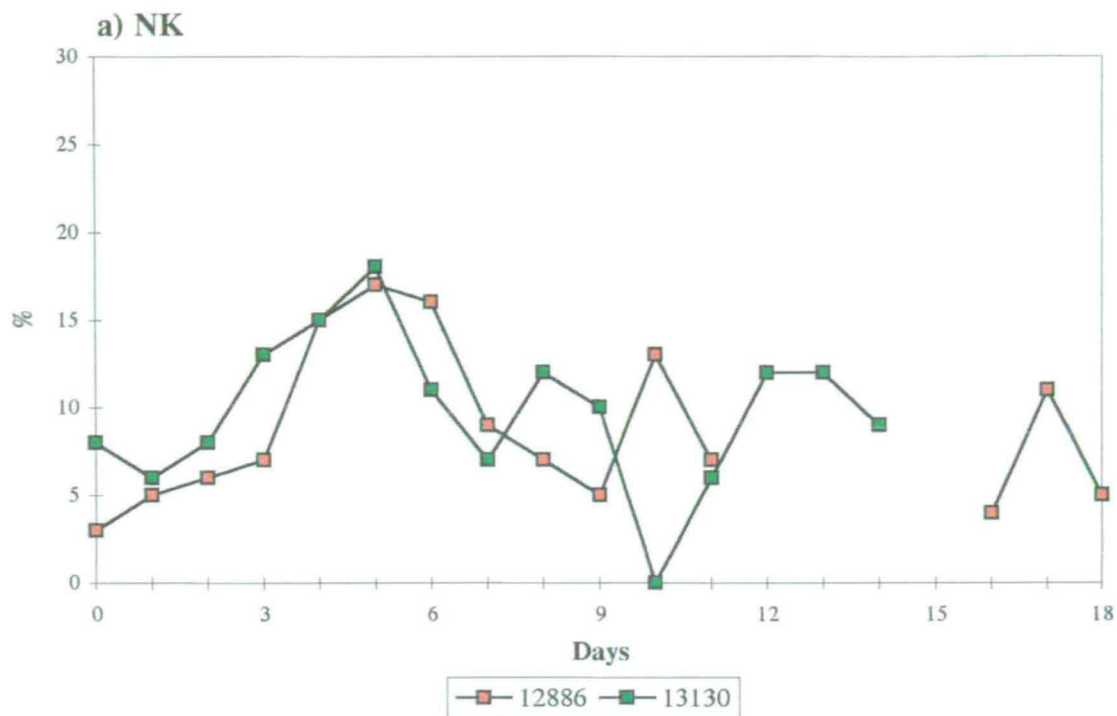
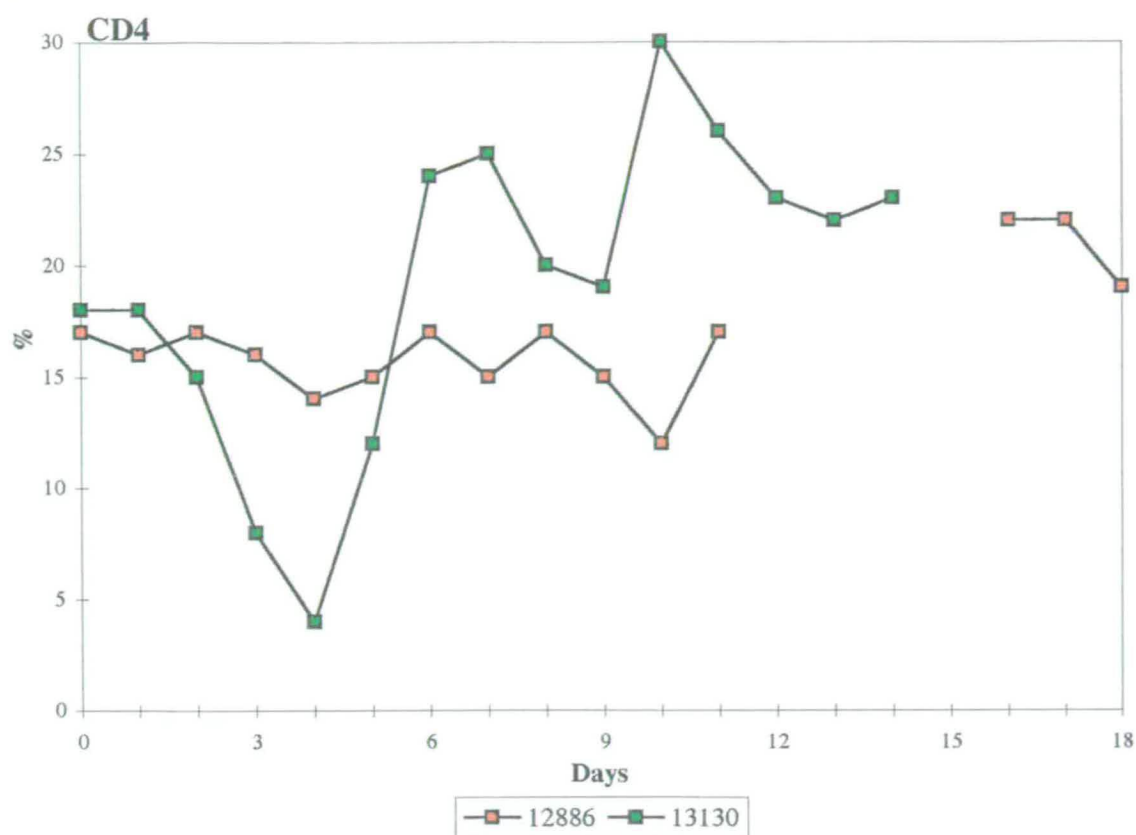


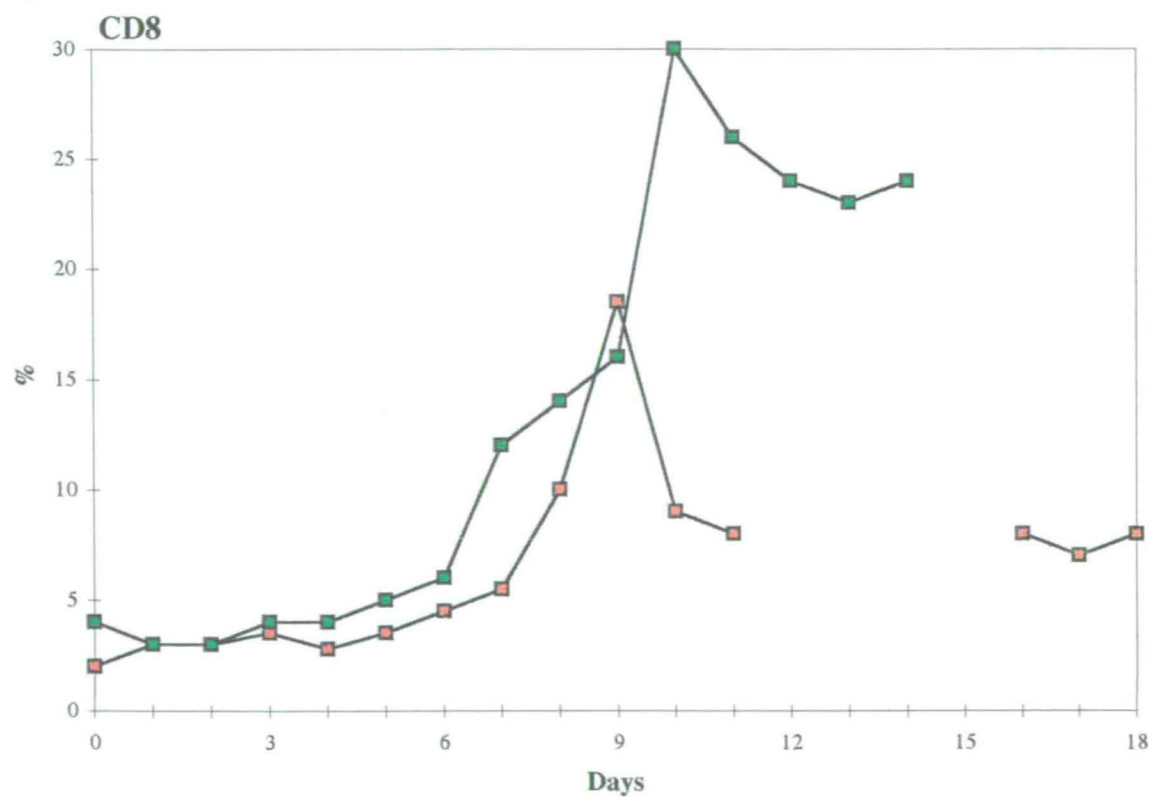
Fig. 7.3 a) Natural killer (NK) and b) CD2 cells in the afferent lymph of calves 12886 and 13130 following immunisation with *T. annulata* (10769) cell line.



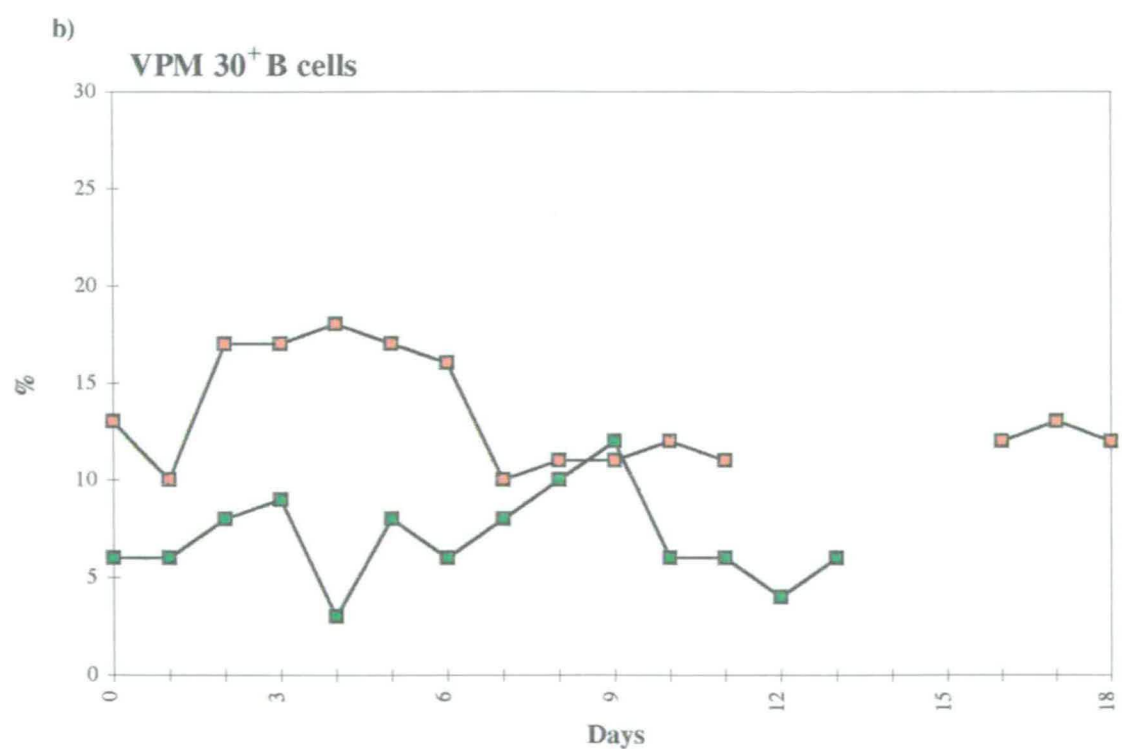
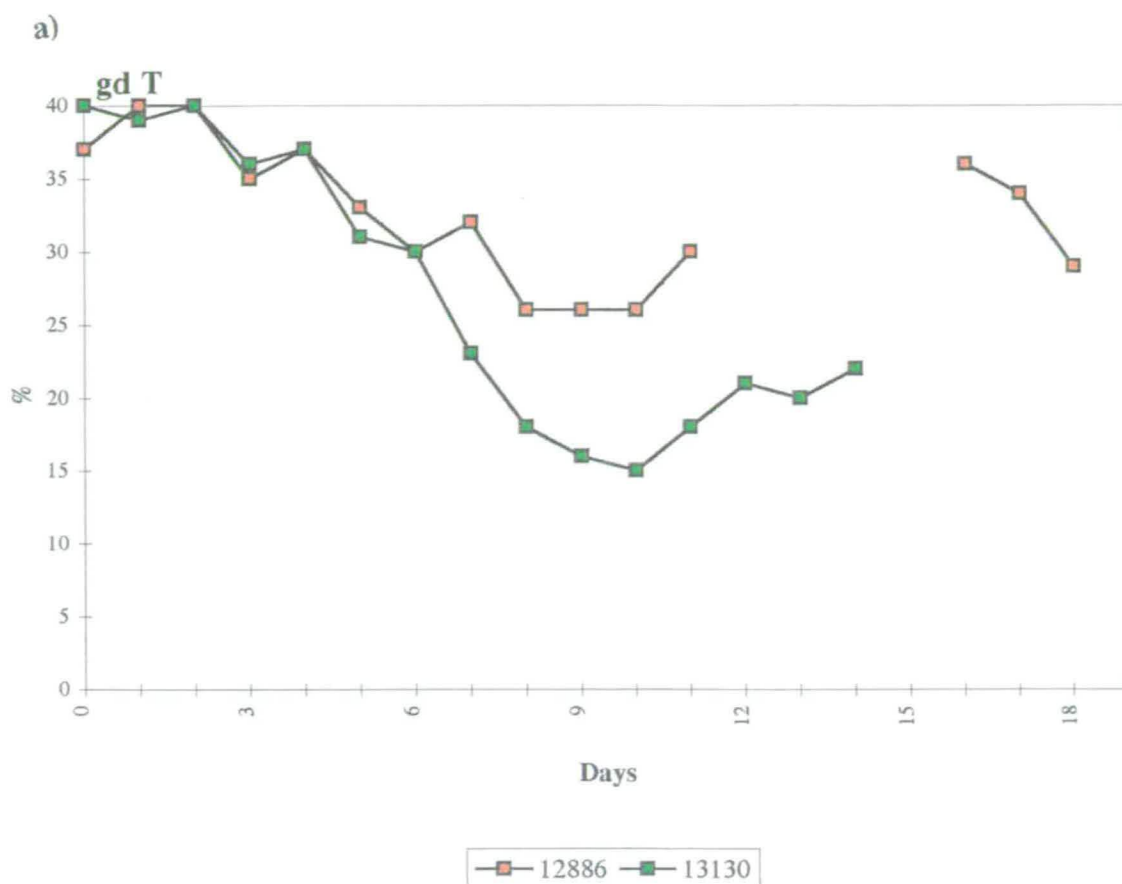
a)



b)



**Fig. 7.4 a) CD4 and b) CD8 cells in the afferent lymph of calves 12886 and 13130 following immunisation with *T. annulata* (10769) cell line. Day 0 represents lymph collected before immunisation.**



**Fig. 7.5 a) Gamma delta (gdT) and b) VPM30<sup>+</sup> B cells in the afferent lymph of calves 12886 and 13130 following immunisation with *T. annulata* (10769) cell line.**  
**Day 0 represents lymph collected before immunisation.**

immunisation. The resting level of CD8<sup>+</sup> cells ranged between 2% to 7% in all animals. A peak level of CD8<sup>+</sup> cells reached 30% and 18% by days 10 and 9 in calves 13130 and 12886 respectively (Fig. 7.4). The levels of  $\gamma\delta$ T cells declined sharply after immunisation in all calves (Fig. 7.5). A small population of 6-13% B cells (identified by mAb VPM 30) was observed on day 0 in two calves which increased to 9% and 18% after immunisation (Fig. 7.5).

#### *7.4.1.2 Kinetics of the nonlymphocyte subpopulations in the afferent lymph*

A wide range of nonlymphocytic cells was found in the two calves ranging between 14-22% in one calf and 13-26% in the other calf. All the VPM65<sup>+</sup> cells were IL-A109<sup>+</sup>. Most of IL-A109<sup>+</sup> cells were IL-A24<sup>+</sup>. 5-10% IL-A109<sup>+</sup> IL-A24<sup>+</sup> cells occurred in both calves (data not shown).

#### *7.4.1.3 Natural killer like cells*

The total number of CD4<sup>+</sup> and CD8<sup>+</sup> cells could not account for the total number of CD2<sup>+</sup> cells. MAb IL-A26 only recognises CD2<sup>+</sup> and mAb CC15 only recognises  $\gamma\delta$ T cells and the  $\gamma\delta$ T cells are CD2<sup>-</sup>. Bovine natural killer cells are CD2<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> (Evans & Jaco-Friedmann, 1993). The NK like cells observed in afferent lymph after immunisation are shown in Fig. 7.3. Maximum number of CD2<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> cells were observed between days 3-6 after immunisation.

#### *7.4.1.4 Activation of T cells in the afferent Lymph*

The expression of activation markers (IL-2R and MHC classII) on CD4, CD8 and  $\gamma\delta$  T cells are shown in Fig. 7.6-7.11. Eight to ten percent activated T (IL-2R<sup>+</sup> CD4<sup>+</sup>) cells were observed in the resting and day 1 afferent lymph of calves. There was no change in calf 12886 while a decrease was noticed, by day 12, in calf 13130. A slight

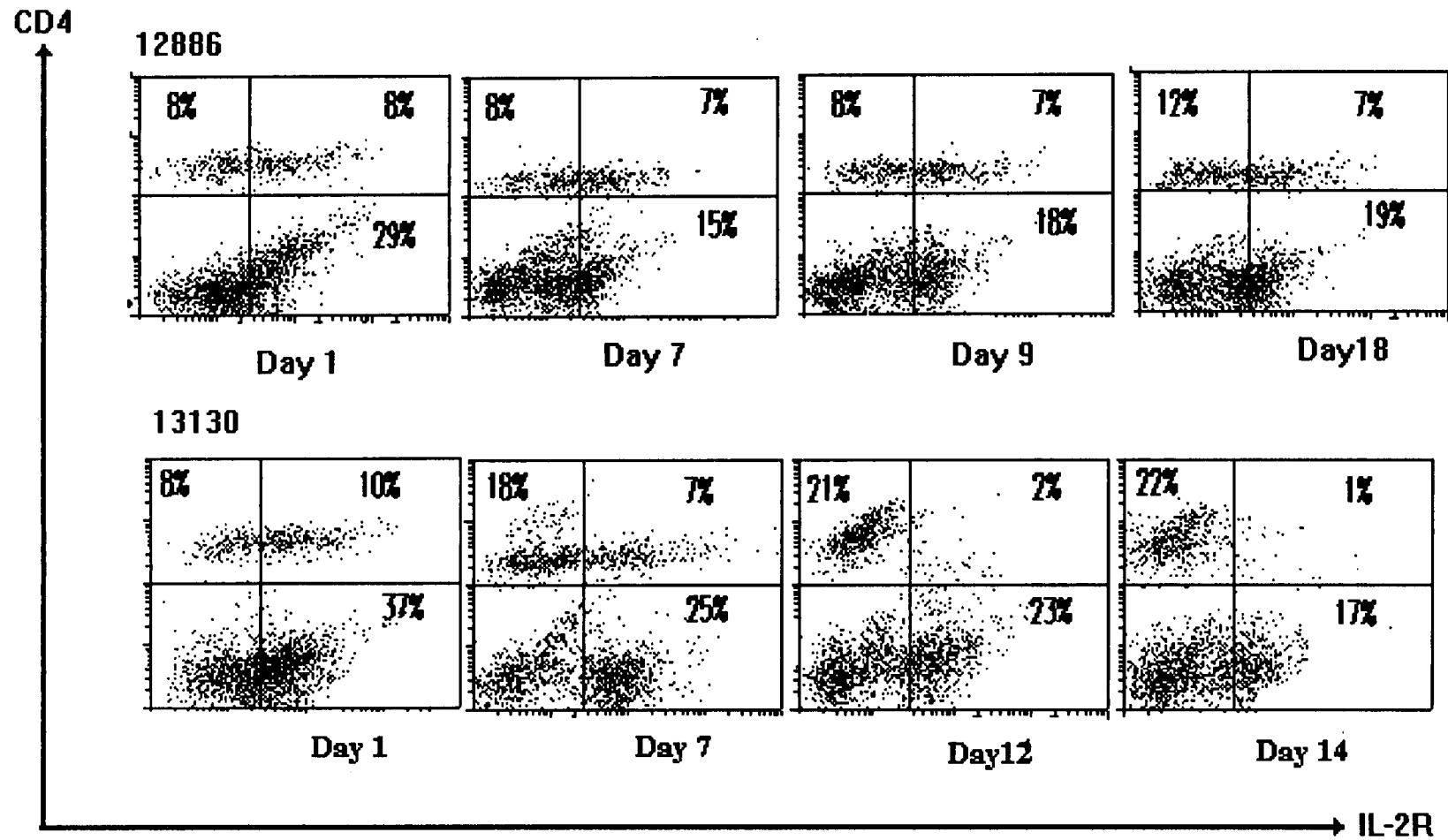


Fig. 7.6 IL-2R expression on CD4 cells in the afferent lymph after immunisation.  
Animals: 12886, 13130

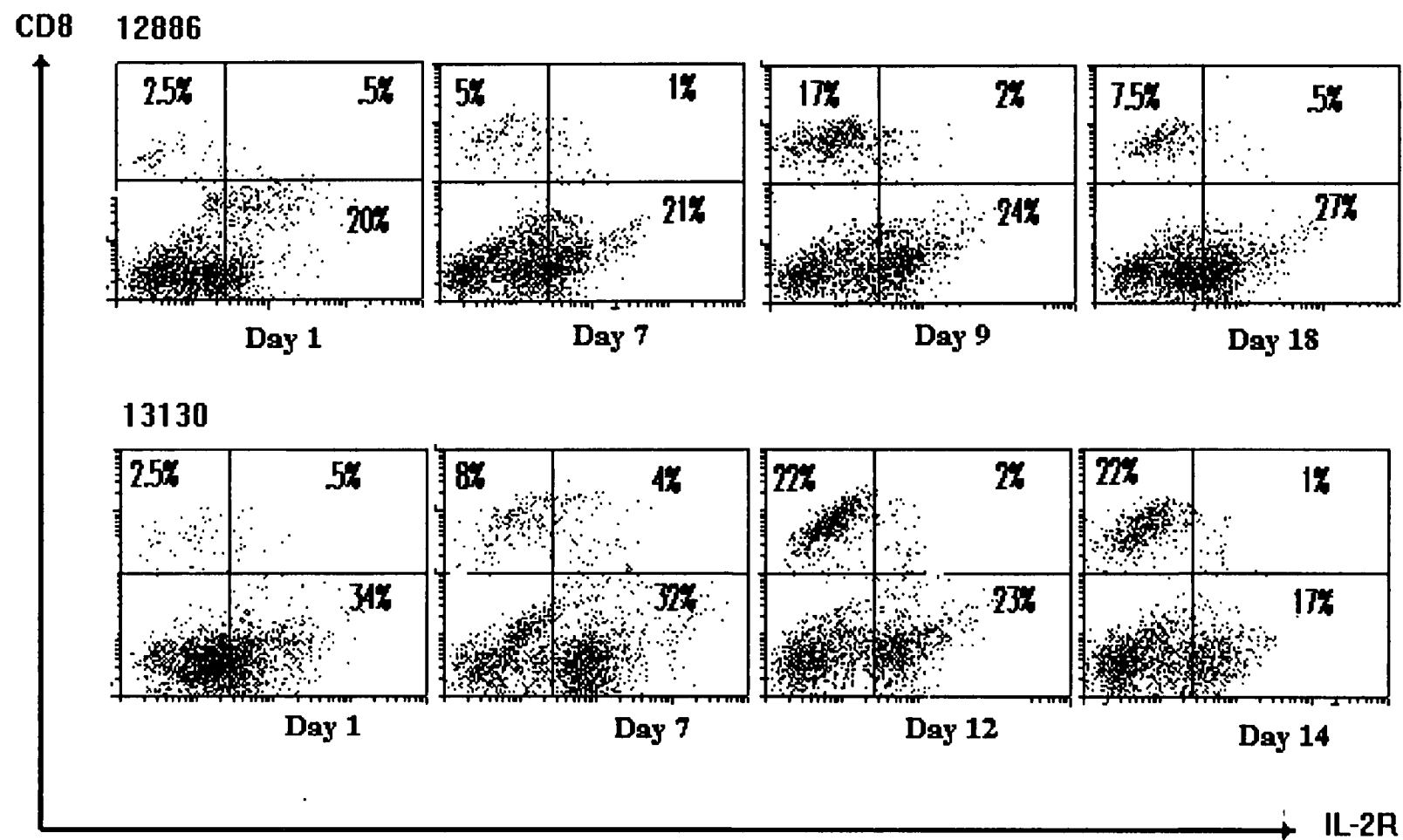


Fig. 7.7 IL-2R expression on CD8 cells in the afferent lymph after immunisation.  
Animals: 12886, 13130

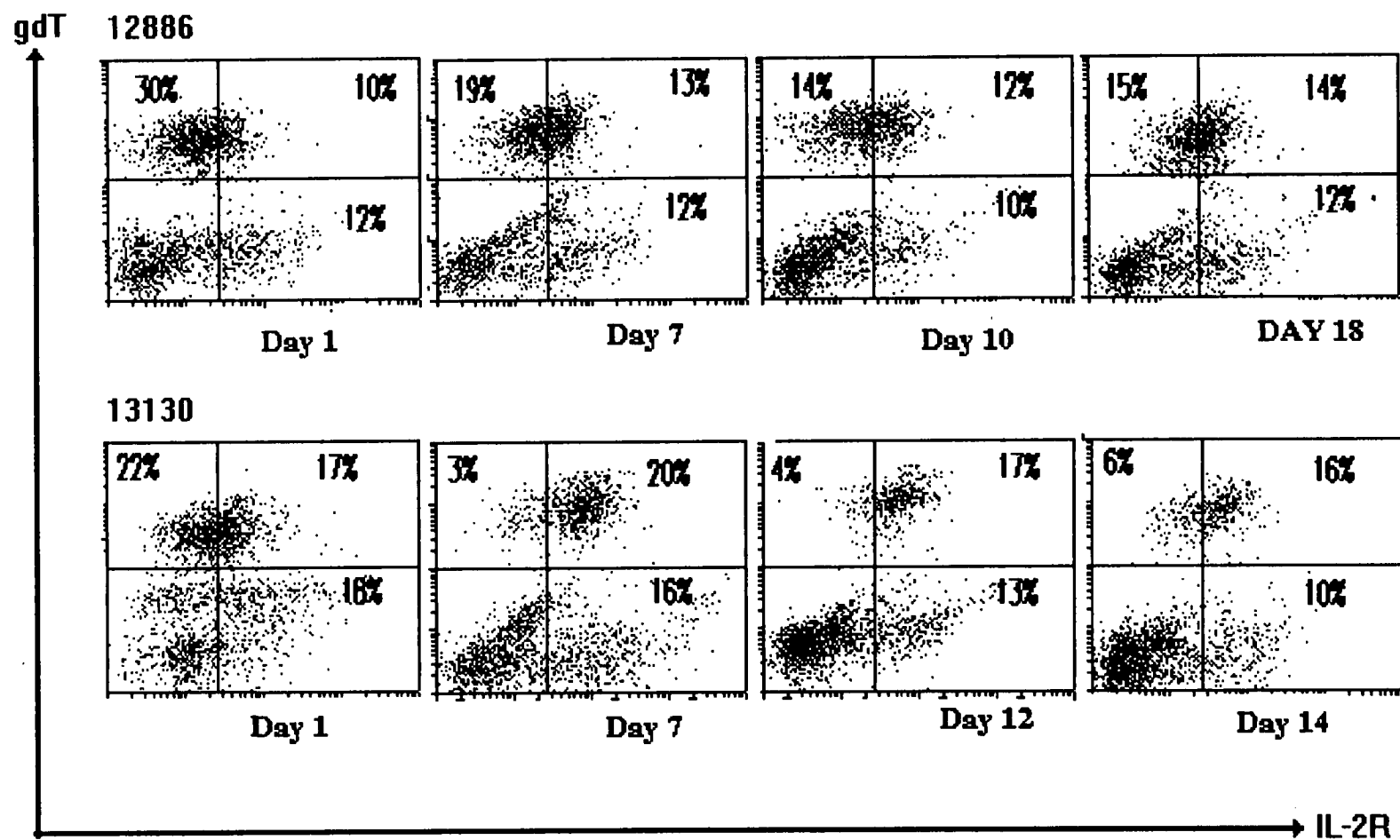
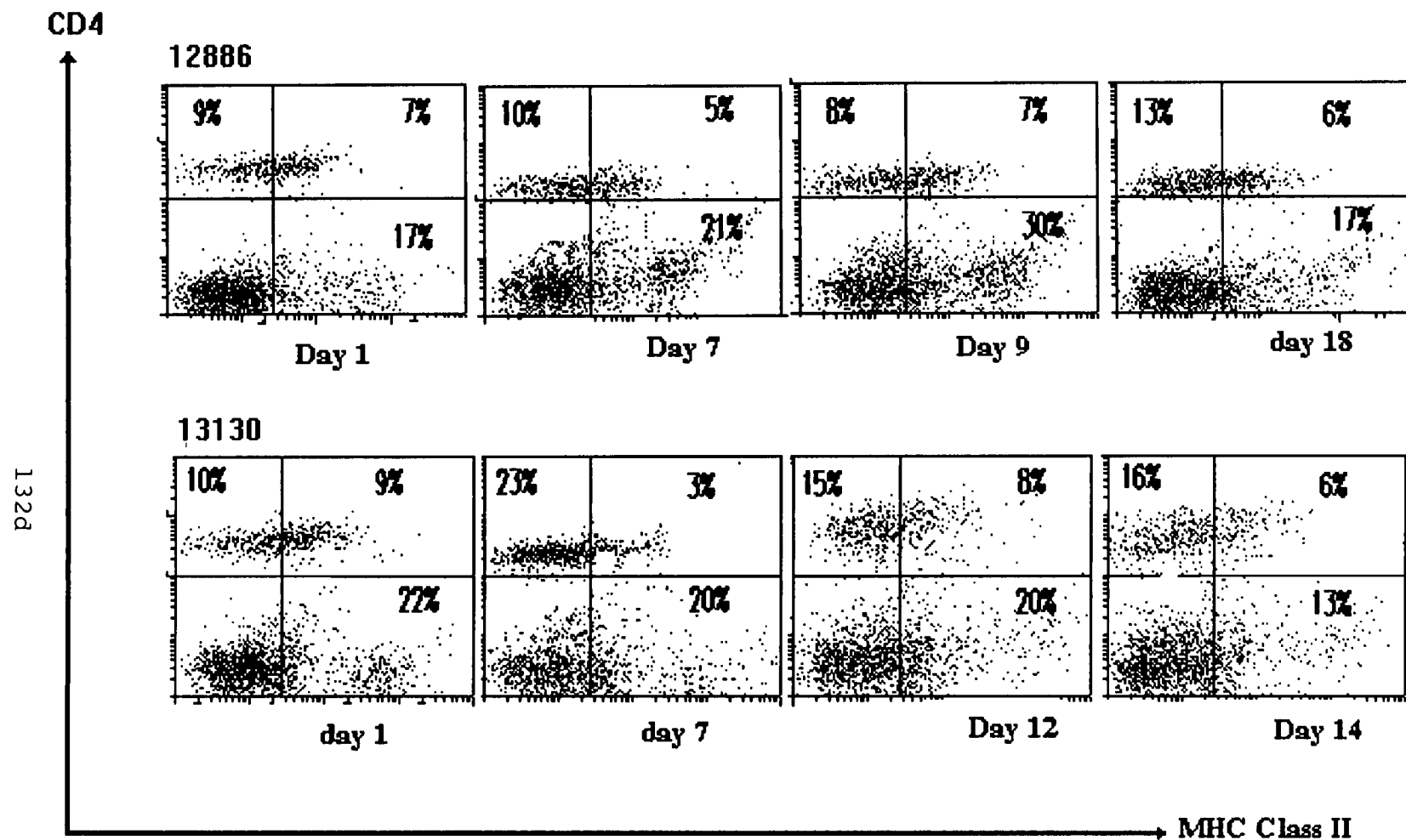


Fig. 7.8 IL-2R expression on gdT cells in the afferent lymph after immunisation.  
Animals: 12886, 13130



**Fig. 7.9** MHC class II expression on CD4 cells in the afferent lymph after immunisation.  
Animals: 12886, 13130

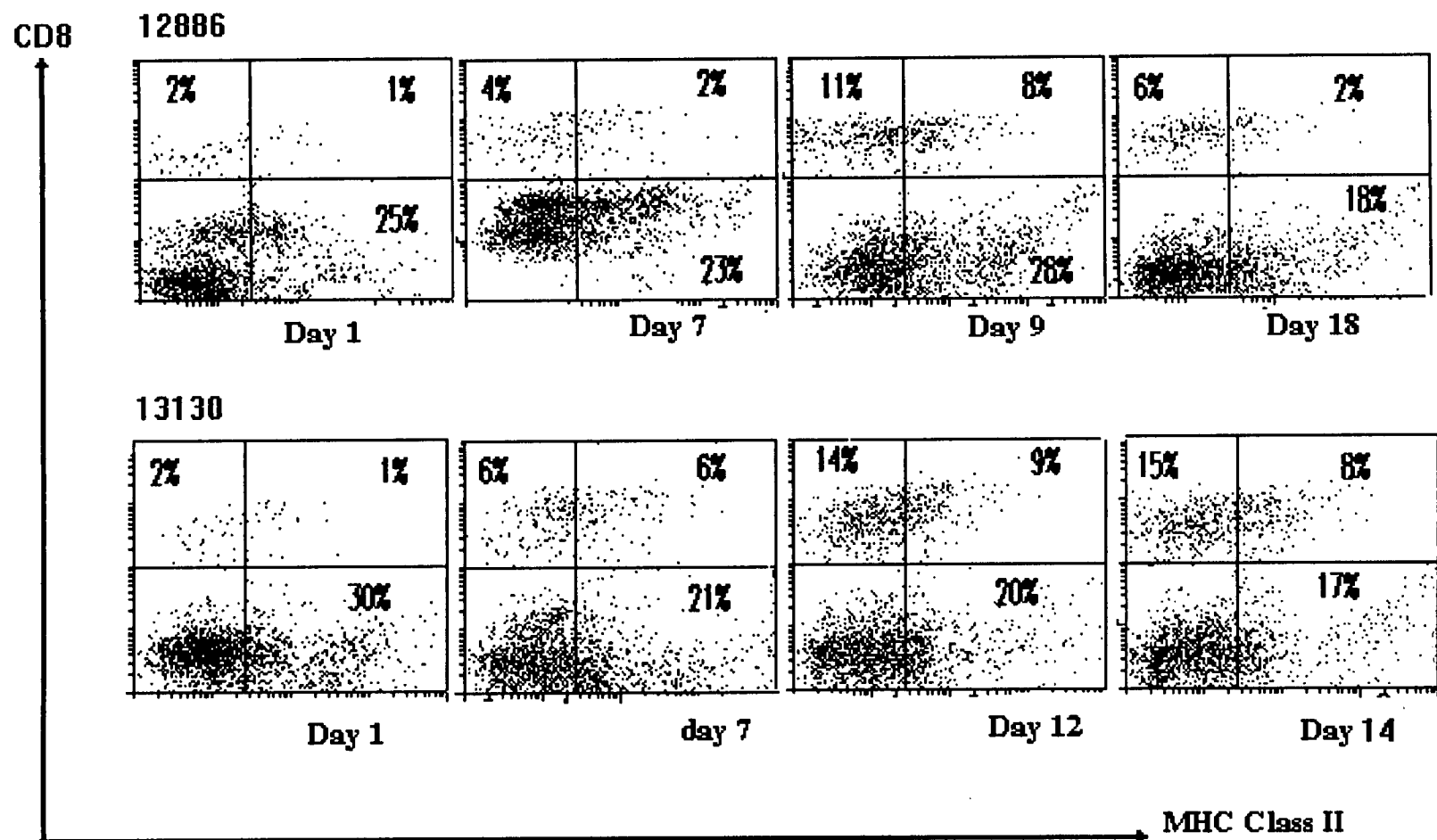


Fig. 7.10 MHC class II expression on CD8 cells in the afferent lymph after immunisation.  
Animals: 12886, 13130



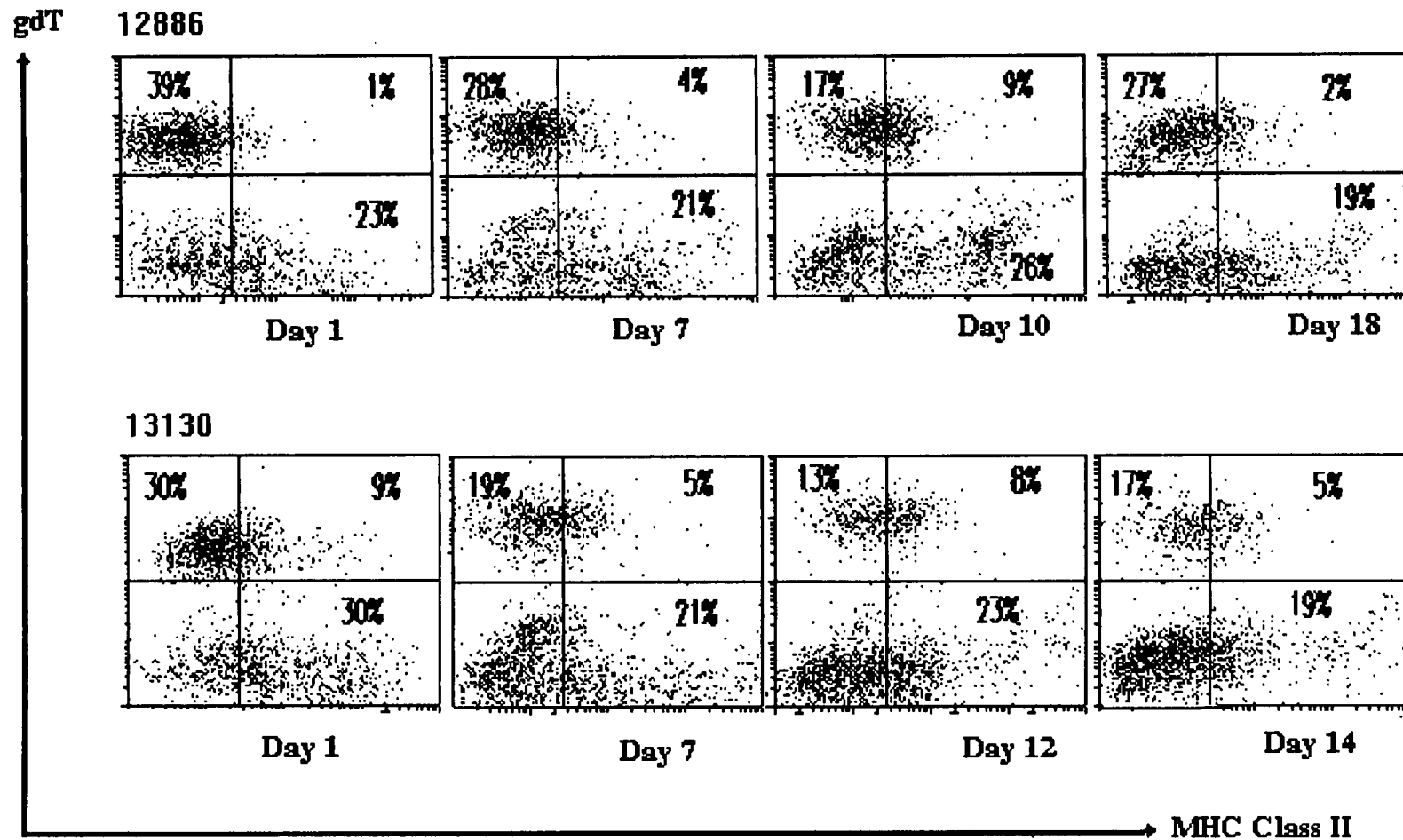


Fig. 7.11 MHC class II expression on gdT cells in the afferent lymph after immunisation.  
Animals: 12886, 13130

increase in the IL-2R expression on CD8<sup>+</sup> cells was observed (Fig. 7.7). At least a third  $\gamma\delta$ T in the resting lymph of all the calves expressed IL-2R (Fig. 7.8). There was little change in the expression of MHC class II on the CD4<sup>+</sup> cells except in one of the calves (fig. 7.9). In contrast, MHC class II expression on CD8 cells started to increase by days 6 and 7 and reached peaks of 8% and 9% by days 9 and 12 in calves 886 and 130 respectively (Fig. 7.10). MHC class II expression increased on  $\gamma\delta$ T cells reaching a peak of 9% and 8% by days 10 and 12 in both calves.

#### **7.4.1.5 Memory markers**

The expression of memory markers CD45RB on CD4, CD8 and  $\gamma\delta$ T cells is shown in Fig. 7.12- 7.14. More than 90% CD4<sup>+</sup> cells were CD45RB "low" effector type i.e. 1-2% of cells expressed high levels of CD45RB (Fig. 7.12). Majority of CD4 cells were CD45RB "low" throughout the post vaccination period except in calf 13130 where the CD45RB "high" population increased to 11% (Fig. 7.12). Most CD8 cells on day 1 were CD45RB "low" but some (8-11%) of them became CD45RB "high" after 9 days after immunisation (Fig. 7.13). An increase in CD8<sup>+</sup> cells was associated with an increase in CD45RB "high" as well as with the MHC class II expression on CD8<sup>+</sup> cells in both calves. 95%  $\gamma\delta$ T cells were CD45RB "low" on day 1 (Fig. 7.14). They remained CD45RB "low" throughout the observation period except in animal 13130 where expression of CD45RB "low" decreased by day 7 but increased again by day 12.

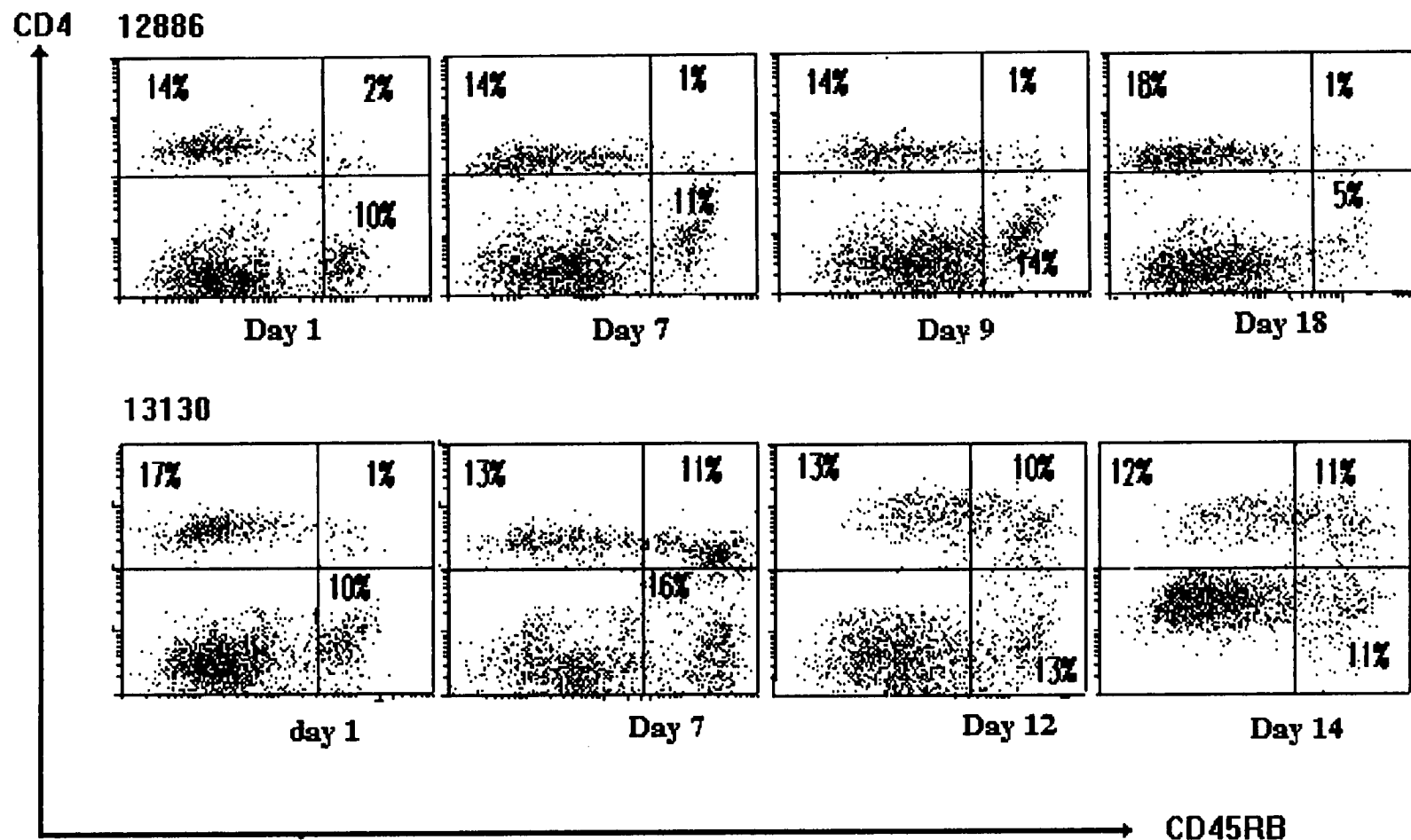


Fig. 7.12 CD45RB expression on CD4 cells in the afferent lymph after immunisation.  
Animals: 12886, 13130

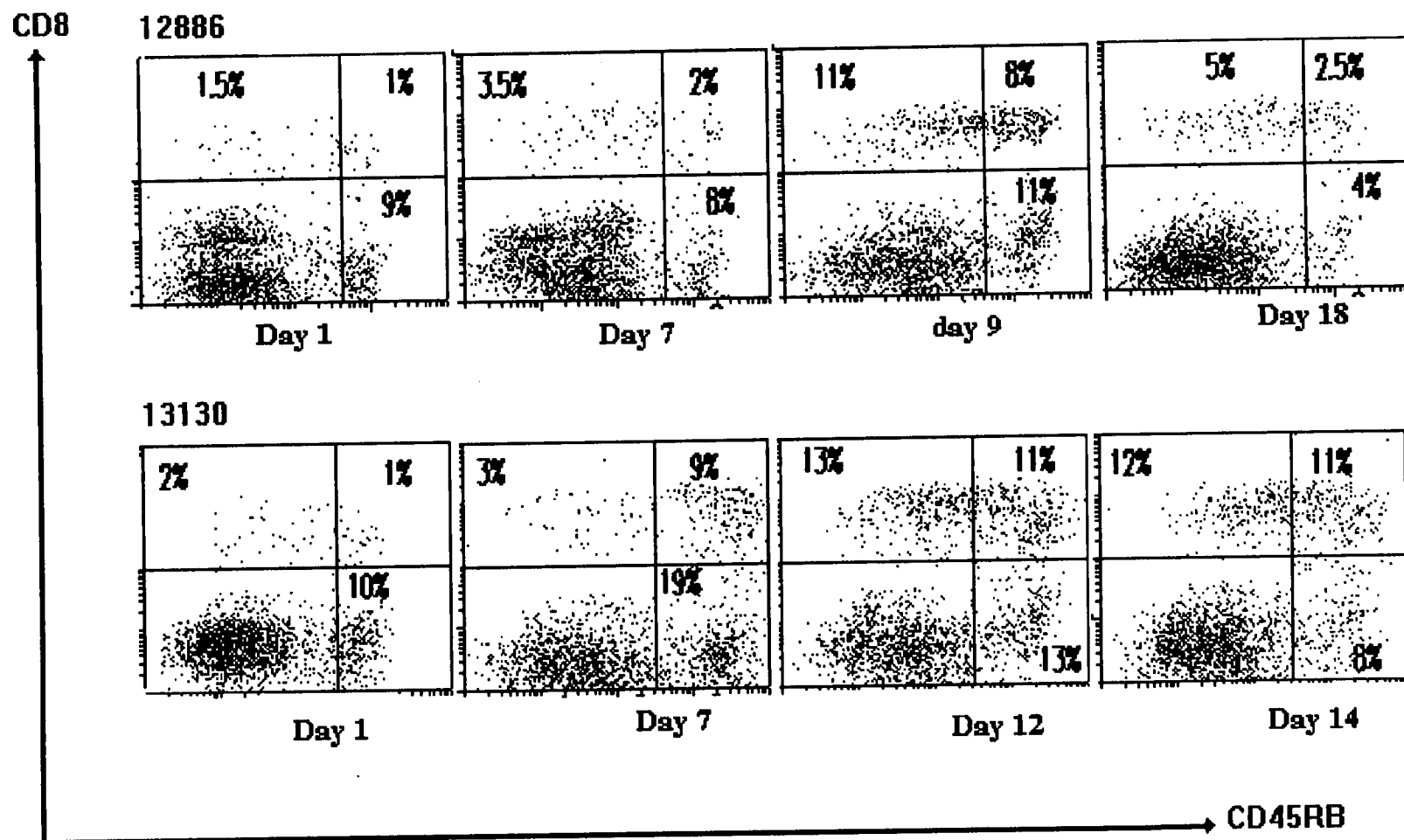
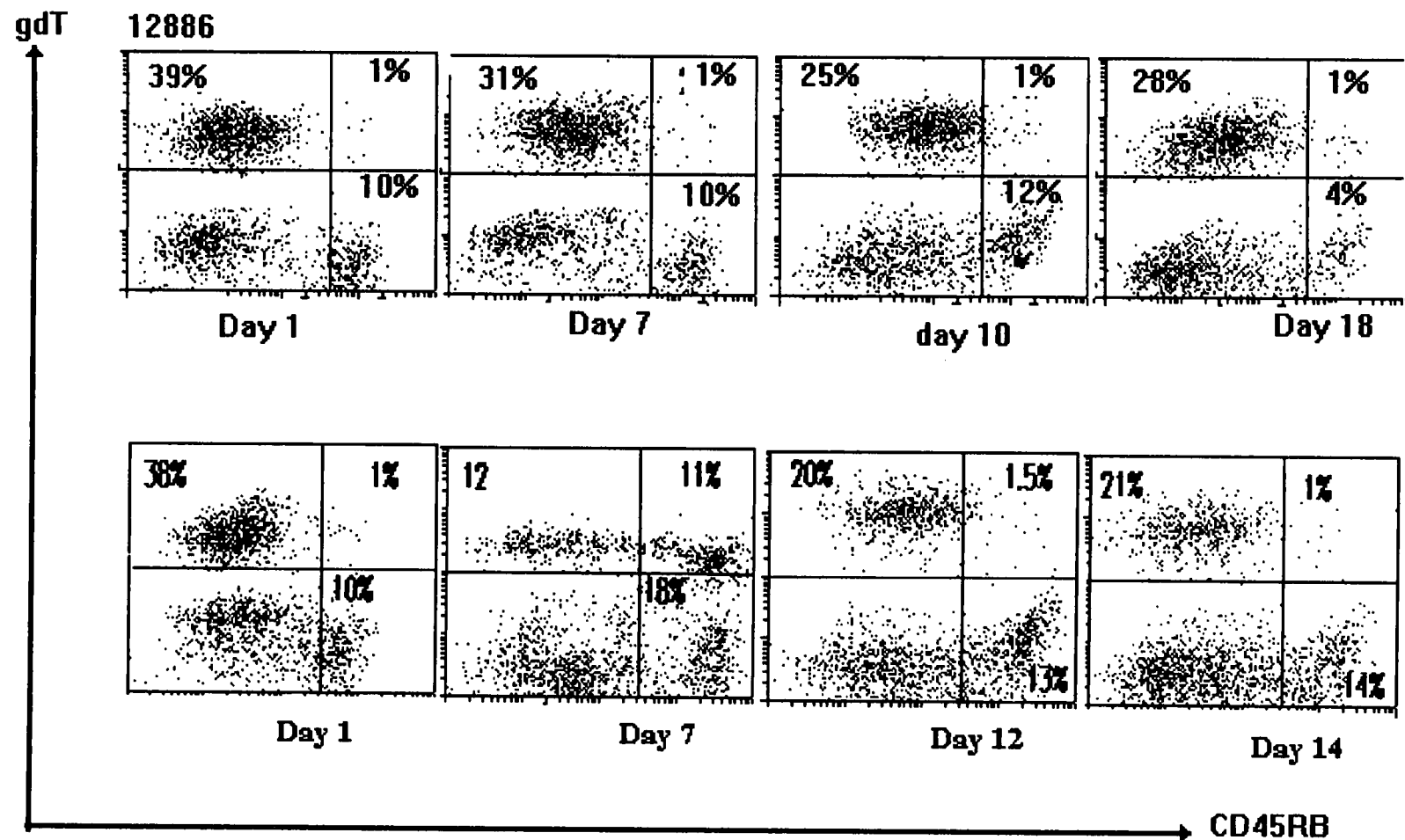


Fig. 7.13 CD45RB expression on CD8 cells in the afferent lymph after immunisation.  
Animals: 12886, 13130



**Fig. 7.14** CD45RB expression on gdT cells in the afferent lymph after immunisation.  
Animals: 12886, 13130

## 7.4.2 Functional assays

### 7.4.2.1 Proliferative responses of afferent cells in in vitro assays

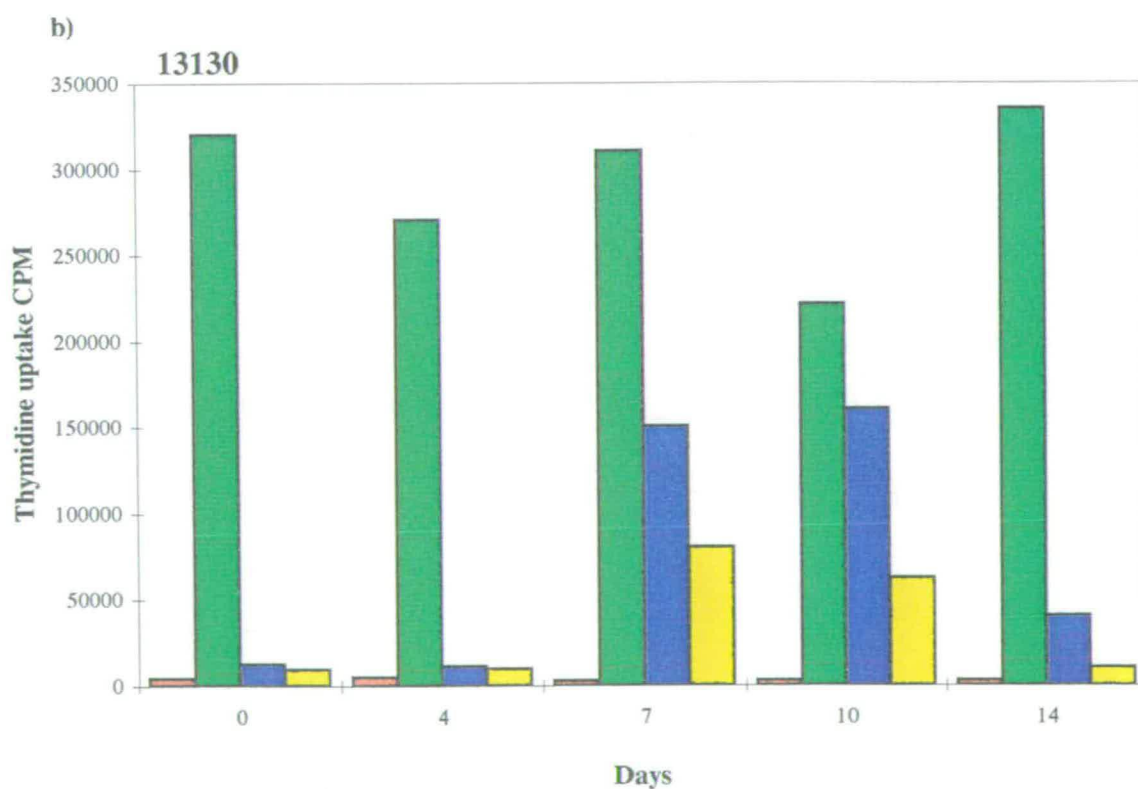
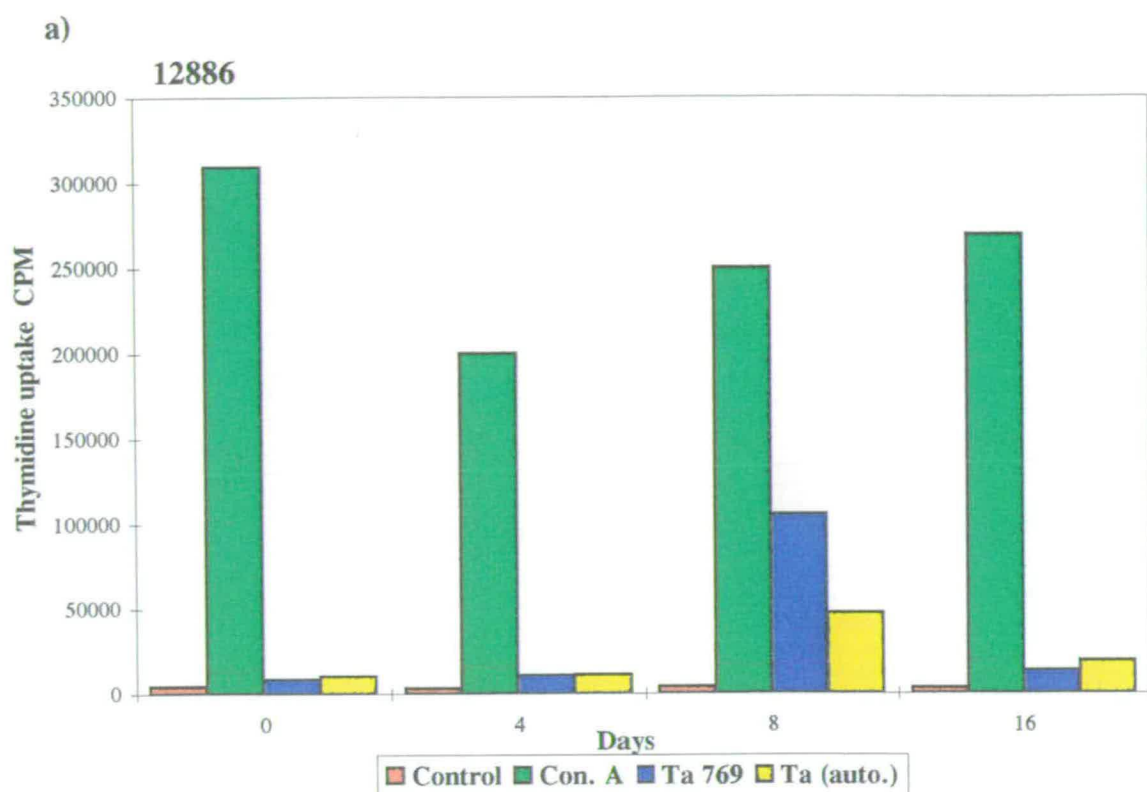
The functional activation state of the lymphocytes draining the site after immunisation of calves 12886 and 13130 (Fig. 7.15) was measured by their proliferative ability in response to incubation with Con A, *T. annulata* infected immunising cells (*Ta* 769) or *T. annulata* infected autologous cells (*Ta auto.*). The control represents afferent cells on their own in the medium. Stimulators were also cultured on their own and their counts have already been deducted from the results shown in Fig. 7.15.

A sharp increase in proliferation in response to the immunising cell line was observed between days 7 and 10 in calves 12886 and 13130 (Fig. 7.15). Increased proliferative responses were also observed in response to the autologous cell line in both calves, but the response was not so strong as with the immunising cell line. A maximum response was observed at days 8 in calf 12886 and 7 in calf 13130.

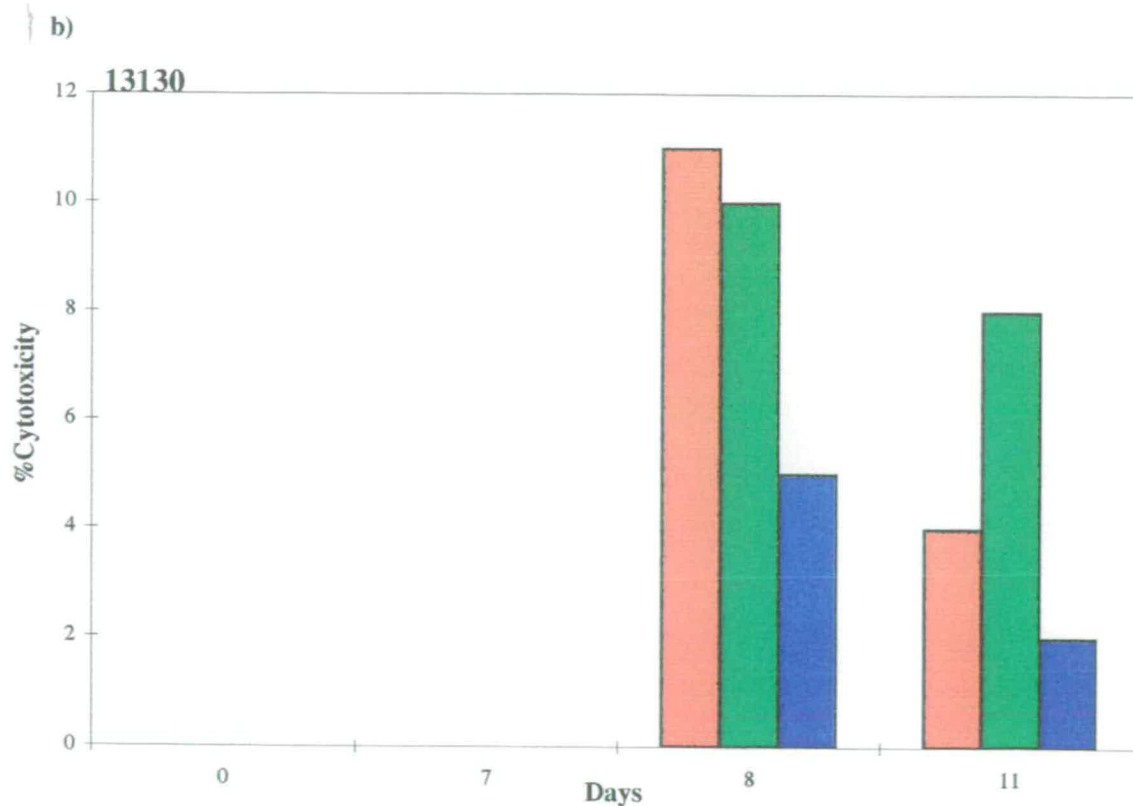
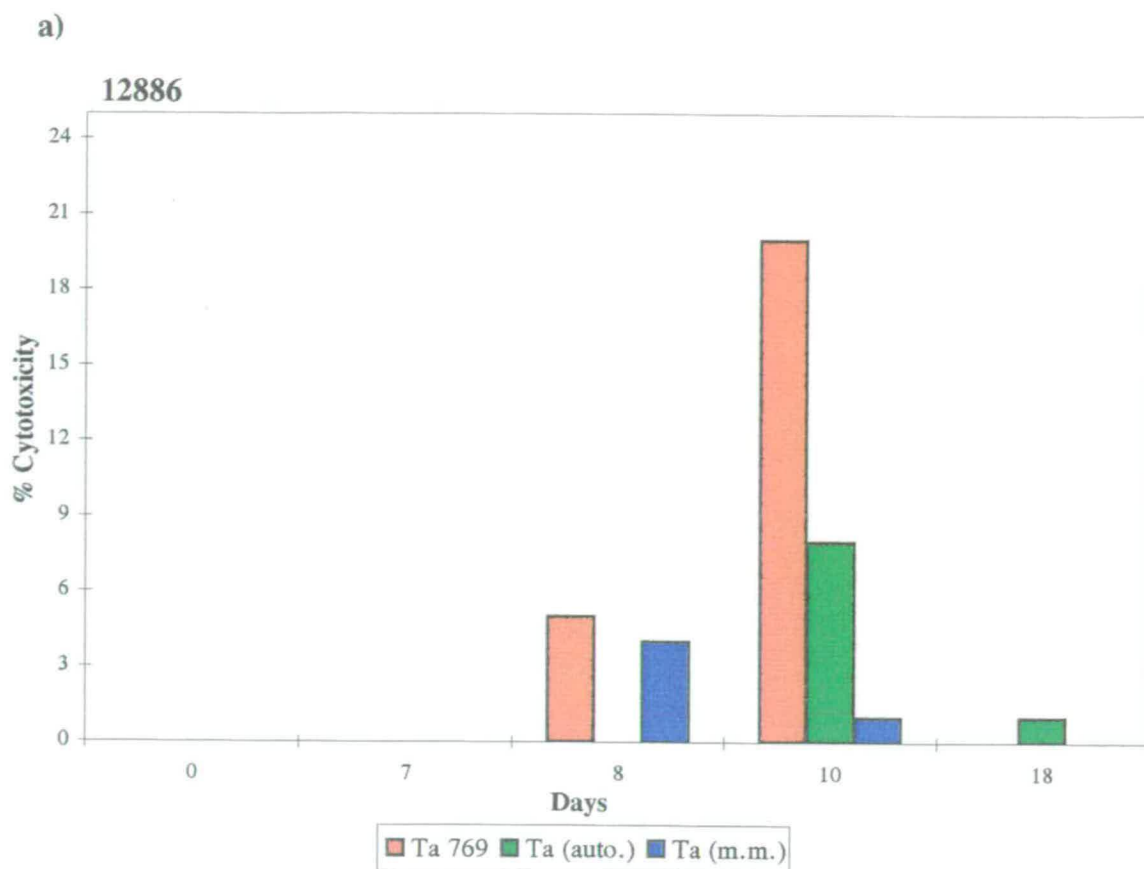
### 7.4.2.2 Cytotoxic responses of afferent cells in in-vitro assays

Cytotoxic responses of the afferent cells were measured against the *T. annulata* infected cells of the immunising cell line (*Ta* 769), *T. annulata* infected cells of the autologous cell line (*Ta auto.*) and *T. annulata* infected cells of totally mismatched cell line (*Ta m.m.*). These assays were performed from day 7 onwards as shown in Fig. 7.16.

Cytotoxic activity against the immunising cell line appeared by day 8 and waned by day 18 in calf 12886. A weak cytotoxic response against the autologous cell line was observed at day 10 which had waned by day 18. Calf 13130 showed weak cytotoxic responses as compared to 12886. Its cytotoxic responses against the immunising cell



**Fig. 7.15** Proliferative responses of afferent lymph cells (ALC) of a) calf 12886 b) calf 13130 incubated with Con.A, *T. annulata* immunising (Ta 769) and *T. annulata* autologous (Ta auto.) cell lines. Results expressed as counts per minute (CPM) by responders ALC. Day 0 represents lymph collected before immunisation.



**Fig. 7.16** Cytotoxic responses of afferent lymph cells (ALC) of a) calf 12886  
 b) calf 13130 incubated with *T. annulata* immunising (Ta 769), *T. annulata* autologous (Ta auto.) and *T. annulata* mismatch (Ta m.m.)  
 cell lines in a 4 hour chromium release assay  
 Results expressed as percent (%) cytotoxicity by ALC.



line and autologous cell line started on day 8 but started to wane by day 11. A very weak response was observed in calves 13130 and 12886, against the totally mismatched *T. annulata* cell line.

#### **7.4.3 Immune status of immunised calves after challenge with sporozoites**

Two immunised calves 13054 and 12886 along with a control naive 12885 were challenged with a potentially lethal dose of 1 t.e. of *T. annulata* (G). All the three animals were without their draining lymph nodes.

##### **7.4.3.1 Clinical reactions**

The immunised calves showed mild parasitological reactions with peak parasitaemia of 0.1% to 0.5%. However the control calf 12885 showed severe parasitological reactions with parasitaemia of 0.1% on day 9 peaking at 10% on day 11. Calf 12885 did not eat sufficient hay for two days but did eat sufficient concentrate. It was treated with 10ml Butalex on day 10. The piroplasms became pyknotic after this treatment and then started to disappear.

There was no rise in temperature in calves 13054 and 12886 but the control calf 12885 showed a rise in temperature by day 7 reaching a peak of 41.7°C on day 10 and started to decline after treatment. The challenge dose and batch was so lethal that the control calf took 10 days before its temperature came down to normal level after treatment with Butalex.

There was small drop in TEC, TLC and PCV in the immunised calves but a sharp decline in all 3 parameters was noticed in calf 12885 by day 10. The PCV declined to 15% by day 14 but started to increase soon after. The animal was treated with 8ml

of vitamin B<sub>12</sub> on alternate days for three days to boost its erythropoietic system.

## 7.5 DISCUSSION

### 7.5.1 Subpopulation of afferent cells and T cell activation

In afferent lymph an increase in CD2<sup>+</sup> cells was associated with an increase in CD8<sup>+</sup> cells and expression of MHC Class II on CD8<sup>+</sup> cells whereas in efferent lymph an increase in CD2<sup>+</sup> cells was associated with an increase in CD4<sup>+</sup> as well as CD8<sup>+</sup> cells (Nichani, 1995). The increase in CD8<sup>+</sup> cells was associated with the decrease in  $\gamma\delta$ T cells suggesting that these  $\gamma\delta$ T cells could be localised in the subcutaneous tissue at the site of immunisation. A drop in  $\gamma\delta$  T cells has been reported in the bovine afferent lymph during infection with *Trypanosoma congolense* where it was associated with an increase in the B cells with no change in the CD2 cells (Flynn *et al*, 1994)

The ratio of CD4:CD8 cells changed in favour of CD8 cells in all the calves. Similar findings have been reported in the efferent lymph after immunisation with 10<sup>5</sup> tachyzoites of the Su8 strain of *Toxoplasma gondii* (Innes *et al*, 1995) and after immunisation with the 10<sup>6</sup> cells of a *T. annulata* cell line. The large increase in the CD8 cells in the efferent lymph resembled the increase in circulating CD8 cells in peripheral blood (Nichani, 1995) and has been reported in animals recovering from *T. annulata* infection (Preston *et al*, 1992a)

Systemic dissemination of the immune response has been attributed to cells exiting in the efferent lymph (Fahy *et al*, 1980; Trinka and Cahill, 1980; Nichani, 1995). For example, the transfer of *T. parva* specific MHC class I restricted cytotoxic CD8 cells from the efferent lymph of the monozygotic twin prevented the development of disease in the naive twin infected with a potentially lethal challenge dose of

sporozoites (McKeever *et al*, 1994). It is possible that the CD8<sup>+</sup> cells in the efferent lymphatics included cells draining down from the site of immunisation as were observed in this study.

In the present study that there was a little increase in the expression of activation markers on CD4 cells, but a marked increase in expression of MHC Class II and a slight increase in the expression of IL-2R on CD8 cells. Similar findings have been reported in the efferent lymph (Nichani, 1995) and in culture of PBM with *T. annulata* infected cells (Campbell, 1995). While IL-2 is believed to increase MHC class II expression on T cells (Tomia *et al*, 1991), in the present study it looked as if the mere expression of IL-2R was sufficient to express MHC Class II on CD8 cells. In others work expression of MHC Class II but not IL-2R on T cell resulted in a defective cytotoxic response in various lentivirus infections (Bird *et al*, 1993). However, this does not appear to hold true in *T. annulata* as the ALC consisting of MHC class II positive T cells with low levels of IL-2R were cytotoxic for the allogeneic cells of the immunising cell line and for the parasitised cells of the autologous cell line. At the same time the proliferative responses of the ALC increased markedly to both the immunising cell line and to the autologous infected cells. These functional assays leave no doubt that the afferent lymph cells were already committed either to the allogeneic antigens of the immunising cell lines or to the parasite antigen of the autologous parasite infected cell line and that ALC therefore acted as memory cells to the recall antigens of immunising cell line. Similar findings in the efferent lymph have been reported after *T. annulata* infected cell line immunisation (Nichani, 1995).

These observations raise the question as to whether activated CD4 cells are essential for the activation of CD8 cells. In this study, animals with low level of IL-2R on CD4 and CD8 cells with high expression of MHC Class II on CD8 were solidly

immune to a potentially lethal dose of *T. annulata* sporozoites. In other *in vivo* studies, an antiviral cytotoxic T cell response in mice required the help of CD4 T cells (Ashman & Mullbacher, 1979; Kast *et al*, 1986). Requirement of CD4 help has also been suggested in sheep after immunisation with *Toxoplasma gondii* (Innes *et al*, 1995). Other studies suggest that CD4 T cell help is not required (Buller *et al*, 1987; Rahemtulla *et al*, 1991; Gill, 1993; Kos, 1993) thus mice depleted of CD4 cells responded to a virus infection by developing an *in vivo* virus specific CTL response and subsequently were immune to a lethal infection. Taking the above reports and my findings together it is possible that the mere presence of activated CD4 cell is sufficient for the expression of CTL responses.

A high expression of IL-2R along with the low expression of MHC class II and CD45RB "low" was observed on  $\gamma\delta$ T cells on day 0 of immunisation. *In vitro*  $\gamma\delta$ T cells assumed an activation state when cultured in medium alone. No evidence was found for their activation due to *T. annulata* infected cells (Campbell, 1995). Further studies are required to examine the role of  $\gamma\delta$ T cells in *T. annulata* infection.

After cell line immunisation, majority of CD4 cells were of the CD45RB low (memory/effector) phenotype in the resting afferent lymph and remained so throughout the observation period after immunisation, with a slight increase in the CD45RB high later on. Up to day 4, the CD45RB low cells were not memory cells for *T. annulata* antigens but cells harvested thereafter proliferated well in proliferation assays indicating they were memory cells for *T. annulata*. An increase in CD8 cells was associated with the CD45RB high population. The presence of naive cells in the afferent lymph was surprising as afferent lymph has been well documented to be the route of circulation for memory cells alone (Mackay *et al*, 1990).

### **7.5.2 Cytotoxic responses**

As shown here ALC proliferated well in response to mitogen before and after immunisation with cell lines whereas their response was greatly reduced by day 10 after lethal sporozoite infection (Nichani, 1995). Together these findings suggest that lymphocytes were immunosuppressed after lethal sporozoite infection but were immunocompetent after immunisation with cell line. Allogeneic immune responses started to appear by day 8, weak cytotoxic responses against autologous parasite infected cells by day 10. Both these responses started to wane by day 18 in one animal and by day 11 in the other animal. A peak cytotoxic response in the efferent lymph has been reported between day 7 to 11 after allogeneic cell line immunisation (Nichani, 1995). These observations raise the question as to why there was a weak response in the afferent lymph. It is possible that once the afferent lymph cytotoxic cells reached the draining lymph node, they received a second priming. The second possibility is that naive T cells circulating to the lymph node through the high endothelial venules were being stimulated and helping to increase the cytotoxic response in the lymph efferent from that node.

Before this experiment was undertaken, it was believed that the immune response develops in the draining lymph node (Nichani, 1995; Chapter 3, 4 & 5 of this thesis). However this experiment indicated that an immune response was initiated at the site of immunisation and presumably potentiated in the draining lymph node. A very weak response was noticed against the totally mismatched cell line which could have been due to the NK cell like activity as cells with the NK phenotype were observed early after immunisation.

In reimmunisation studies, preexisting allogeneic responses were reported to be

responsible for the failure of immune response after second vaccination with the same cell line (Nichani, 1995). It is possible that the allogeneic cytotoxic response at the site of immunisation (observed in this study) killed the cell line at the site and thereby prevented the transfer of the parasites to the recipients' cells leading to failure of generation of immunity after second vaccination.

### **7.5.3 Natural killer like cells**

CD2<sup>+</sup> CD3<sup>-</sup> CD4<sup>-</sup> CD8<sup>-</sup> cells were observed during 3-6 days after immunisation. These cells are believed to be bovine NK like cells (Cook *et al*, 1989; Evans & Jaso-Friedmann, 1993). NK like cells expressing CD45RO at days 5 & 7 after stimulation were reported in cultures of PBM with *T. annulata* infected cells (Campbell, 1995). The expression of CD45RO on CD2<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> (NK) cells as recorded for bovine NK cells (Campbell, 1995) is believed in humans to be due to the activation of these cells (Shen *et al*, 1995). NK cells acquire the activated phenotype only upon incubation with IL-2 or IFN $\alpha$  or IFN $\gamma$  (Jenson & Schultz, 1990). Although CD45RO expression was not analysed in this chapter, a weak cytotoxic response was found against a mismatched *T. annulata* infected cell line. As NK cells act in a non-MHC restricted manner, this response could be due to the NK cells in the afferent lymph. Similarly a non-MHC restricted cytotoxicity was observed in *T. parva* and was suggested to be due to the NK cells (Pearson *et al*, 1979 & 1982).

In summary, this chapter described the activation of T cells at the site of immunisation with cell lines including a weak cytotoxic response against allogeneic antigens as well as against parasite antigens. The large number of NK cells detected by FACS analysis during 3-6 days after immunisation, strongly suggested a role for these cells during the first week of immunisation. To confirm this, and as described

in the next chapter, cannulation experiments were carried out paying particular attention to CD45RO expression on  $CD2^+ CD4^- CD8^-$  NK cells. As the expression of the activated NK like cell phenotype is believed to be due to  $IFN\gamma$ , the biological activity of  $IFN\gamma$  in the afferent lymph was also examined.

## **CHAPTER 8**

### **KINETICS OF NATURAL KILLER LIKE CELLS IN THE AFFERENT LYMPH IN RESPONSE TO *THEILERIA ANNULATA* INFECTED CELL LINE IMMUNISATION**



## 8.1 INTRODUCTION

In cattle, natural killer (NK) cells activity has been demonstrated against herpes virus (Cook *et al*, 1989; Cook & Splitter, 1989a) and has been shown to be influenced by cytokines (Jensen & Schultz, 1990). However, little is known about NK cells in theileriosis. A weak non-MHC restricted cytotoxicity was observed in the PBM of immune cattle upon *in vitro* stimulation with *T. parva* infected cells and was attributed to NK cells (Pearson *et al*, 1979; 1982). Similar findings were observed in the efferent lymph and PBM of cattle undergoing or recovering from lethal *T. parva* infection (Emery *et al*, 1981a). Similarly, a weak non MHC restricted cytotoxic response was reported in the PBM after *T. annulata* infected allogeneic cell line immunisation (Innes *et al*, 1989a). In addition, a nonspecific protective response against theileriosis was observed after immunisation with *Corynebacterium parvum* (Manickam *et al*, 1983). Although no specific evidence for the involvement of NK cells in protection was obtained, it is possible that NK cells were activated nonspecifically in response to *C. parvum*. Finding cells with weak non-MHC cytotoxicity in the afferent lymph (chapter 7) suggested that NK like activity may occur at the site of inoculation of cell lines.

In species other than cattle, activated NK cells have been shown to be major producers of IFN $\gamma$  (Scharton *et al*, 1993; Sher *et al*, 1993; Cardillo *et al*, 1996) and IL-12 has been reported to be the major stimulus of NK cell activation (Kobayashi *et al*, 1989; Gately *et al*, 1991; Chan *et al*, 1991; D'Andrea *et al*, 1992).

Taken together the above reports of non-MHC restricted cytotoxicity in the *Theileria* infections and *in vitro* observations that IFN $\gamma$  can inhibit the establishment of trophozoite-infected cells (Preston *et al*, 1992b) and proliferation of macroschizont-

infected cells (Nichani, 1995) indicate that NK cells could serve as the first line of defence against *T. annulata*. The experiments described here were therefore undertaken to look for NK cells and IFN $\gamma$  at the site of inoculation of *T. annulata* cell lines.

## **8.2 EXPERIMENTAL DESIGN**

Calves were immunised with an allogeneic *T. annulata* cell line and cannulated after immunisation to provide cells from pseudoafferent lymph draining the site of inoculation. The following analyses were carried out.

- 1) Populations of NK like cells were looked for by flow cytometry. Since no single mAb against bovine NK cells was available a number of mAbs were used to analyse the NK like cells. The CD2<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD45RO<sup>+</sup> cells were taken to be activated NK like cells.
- 2) NK cell like activity was assessed in a 4 hour chromium release cytotoxicity assay using mismatched target cells.
- 3) IFN $\gamma$  was assessed in the afferent lymph by ELISA. Its activity curve was plotted by using a Bo-rIFN $\gamma$  of known concentration.

## **8.3 MATERIALS AND METHODS**

### **8.3.1 Animals**

Two young calves (13144, 13470) of 2-3 months of age were used in this study. Their prescapular lymph nodes were removed 8 weeks before they were immunised with an allogeneic *T.annulata* infected cell line. Their pseudoafferent lymphatics were cannulated 2 days after immunisation. Clinical and parasitological observations were carried out as described (chapter 3).

### 8.3.2 Phenotypic analysis and cytotoxic assay of ALC

Afferent lymph cells (ALC) collected overnight were examined by flow cytometry and assessed in the cytotoxicity assays as described in detail in chapter 7 (7.3.2.1). Since no single mAb for bovine NK was available, the phenotype of NK cells was examined by flow cytometry using mAbs for CD2, CD4, CD8,  $\gamma\delta$ T, CD45RB and CD45RO as described in chapter 7 (7.3.1). Activated NK cells are taken to be CD2<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD45RO<sup>+</sup>. Because the mAbs which recognise CD4, CD8 or CD2 were IgG<sub>2a</sub> (IL-12, CC8), IgG<sub>2a</sub> (SBU-T8) and IgM (IL-A26), the mAb IL-A150 which is IgG isotype could not be used to assess CD45RO<sup>+</sup> on CD4 and CD8 cells. MAb CC76 which is IgG<sub>1</sub> and recognises CD45RB was used instead to assess the memory markers on CD4 and CD8 cells. The use of CD45RB as a memory marker for CD4 and CD8 and of CD45RO as a memory marker for CD2 cells was justified by an earlier observation (Campbell, 1995). In this work, it was observed that the percentage population of CD2<sup>+</sup>CD45RO<sup>+</sup> cells in PBM equalled the sum of the CD4<sup>+</sup>CD45RB low and CD8<sup>+</sup>CD45RB low cells, where low expression of CD45RB was accepted to equate with memory. The number of activated (memory) NK cells was calculated as follows:

$$\text{CD2}^+\text{CD4}^-\text{CD8}^-\text{CD45RO}^+ = \text{CD2}^+\text{CD45RO}^+ - (\text{CD4}^+\text{CD45RB low} + \text{CD8}^+\text{CD45RB low})$$

where CD2<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD45RO<sup>+</sup> are memory (activated) NK, CD2<sup>+</sup>CD45RO<sup>+</sup> are memory (activated) CD2 (including CD4 and CD8), CD4<sup>+</sup>CD45RB low are memory (activated) CD4 and CD8<sup>+</sup>CD45RB low are memory (activated) CD8.

### 8.3.3 Interferon- $\gamma$ assay

For the estimation of IFN- $\gamma$  in the afferent lymph, an aliquot of lymph collected in the morning was centrifuged at 300g for 15 min. and the

cell-free supernate decanted for storage at -20°C. A solid phase sandwich ELISA using mAbs specific for bovine IFN- $\gamma$  was used (Wood *et al*, 1990; Rothel *et al*, 1990) to measure the levels of IFN- $\gamma$  in the lymph plasma using a ready made commercial test kit (CSIRO, Australia). Briefly, the frozen lymph supernates and the antibody coated 96 well plate were thawed and brought to room temperature. 50 $\mu$ l of lymph and the 50 $\mu$ l of positive as well as the negative controls were added to the required number of wells, and the plates were incubated for 1 hour at room temperature. After incubation the plate was washed and incubated with 100 $\mu$ l of conjugate for 1 hour. The plate was washed as above and incubated for 30 minutes with 100 $\mu$ l of substrate. After incubation, 50 $\mu$ l of enzyme stopping solution was added to the wells and the plates were read in an ELISA reader (MR 700 Microplate reader, Dynatech) at 450 nm within 20 min. after adding the final solution to stop the enzyme substrate reaction. Serial two fold dilutions of recombinant bovine IFN- $\gamma$  (Ciba Giegy; a gift from Dr. Gary Entrican, Moredun Institute, Edinburgh) were used to plot a standard curve for estimating the biologically active IFN- $\gamma$  in the lymph samples. All the necessary reagents and procedures were provided with the kit. Each sample was tested in duplicate and the mean O.D. was used to calculate the amount of IFN- $\gamma$ .

## **8.4 RESULTS**

### **8.4.1 Clinical observations**

The temperature, TLC, TEC, PCV and piroplasms were recorded in all the immunised calves. A decrease in lymph flow and cell concentration was noticed. All these clinical observations resembled those observed in cattle described in chapter 6.

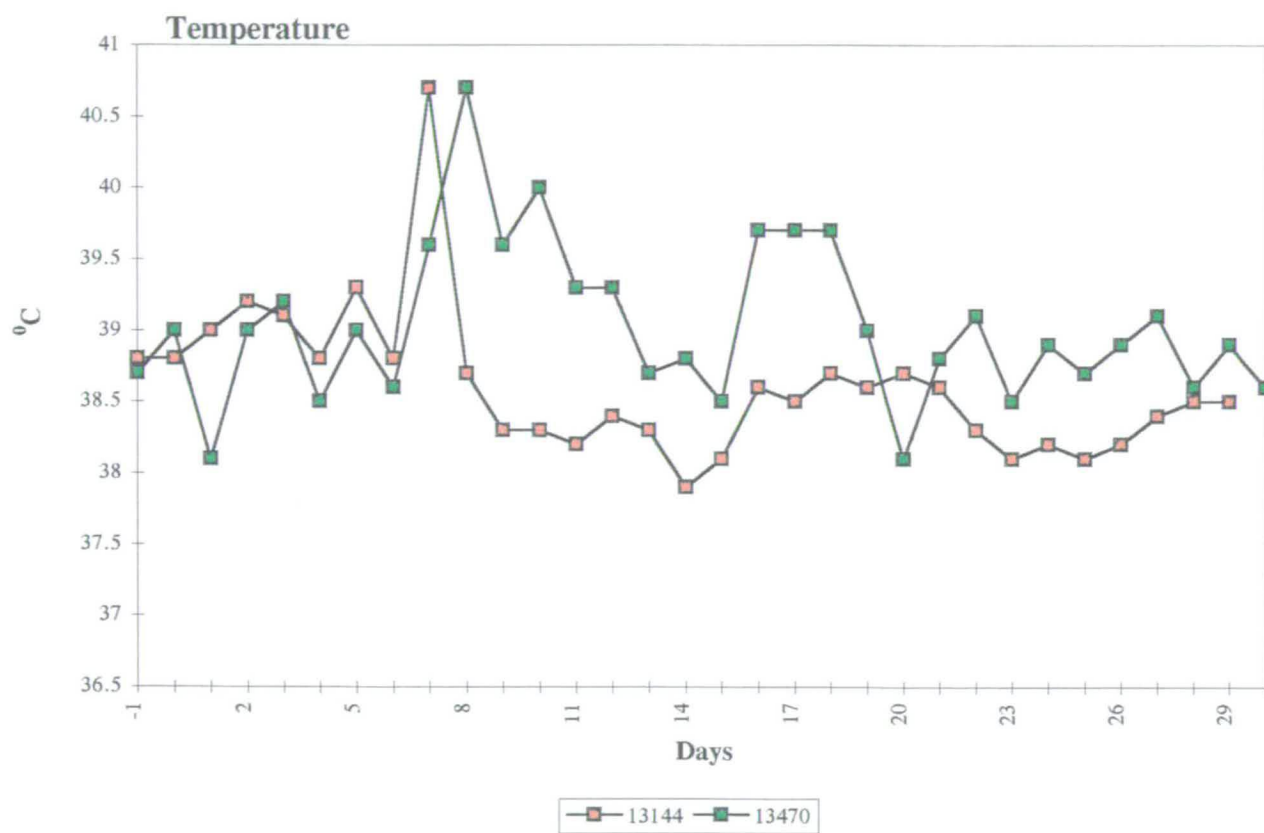


Fig. 8.1 Temperature of calves 13144 and 13470 following immunisation with *T. annulata* (10769) cell line.

#### **8.4.1.1 *Temperature, TLC, TEC and PCV***

A single peak of temperature (40.7°C) was observed in calf 13144 on day 7 while a peak (40.7°C) occurred on day 8 in calf 13470. The second peak (39.7°C) was observed between days 16-18 in calf 13144 (Fig. 8.1). TLC, TEC and PCV decreased as observed in cattle described in chapter 6 (data not shown).

#### **8.4.1.2 *Parasitological observations***

Piroplasm parasitemia of 0.1% and 0.3% were noticed on days 7-13 and 16-18 in calves 13144 and 13470 respectively.

### **8.4.2 *Flow cytometric analysis***

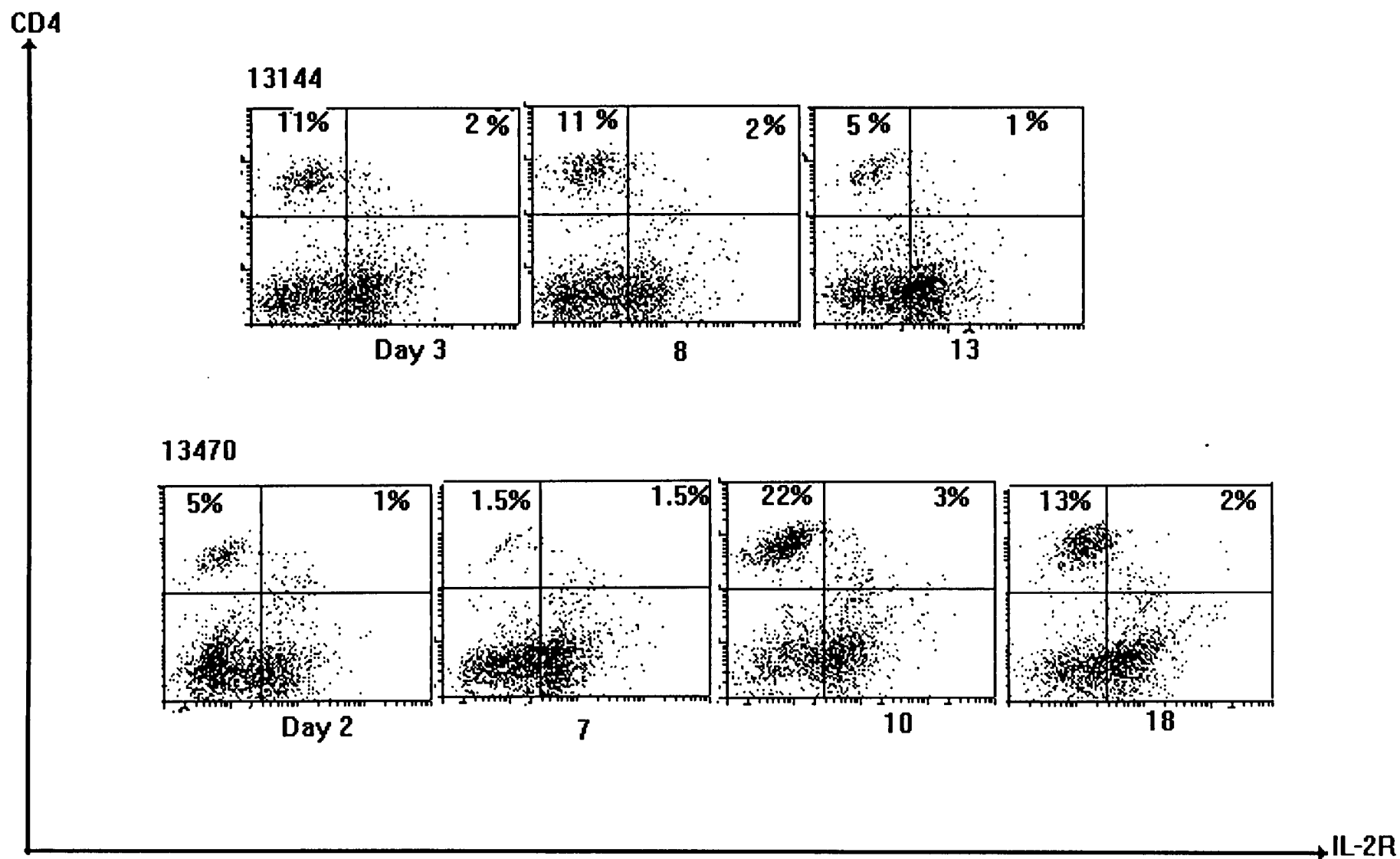
#### **8.4.2.1 *Kinetics of CD2, CD4 and CD8 cells***

A total of 15-34% CD2 T cells were observed on day 2. Six percent CD4 and CD8 cells were found in 13470 and 12% in 13144 on day 2 and 3 after immunisation. CD4 and CD8 cells increased to 23% in 13470 with no increase in 13144 (data not shown).

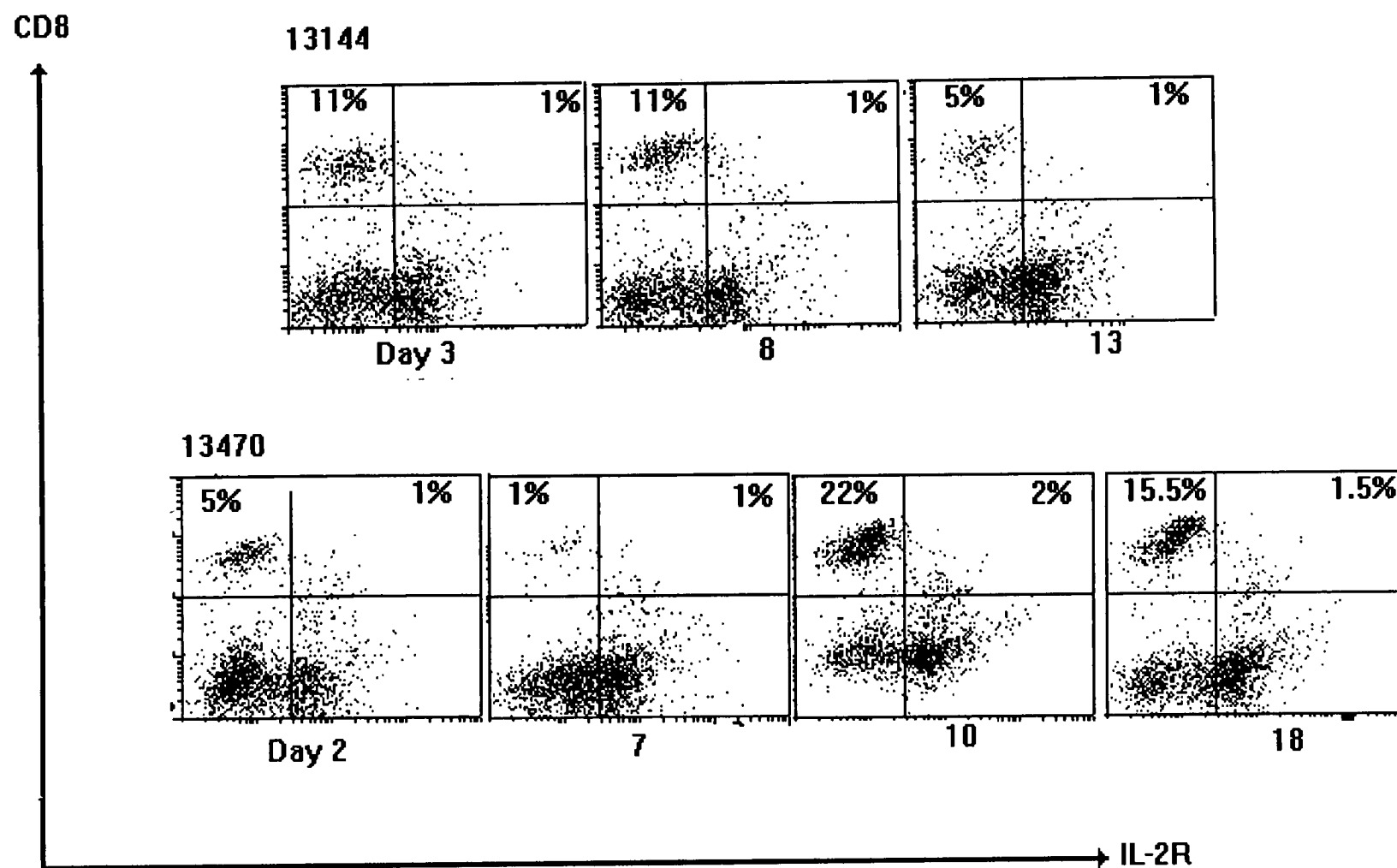
#### **8.4.2.2 *Activation markers***

The results of two calves were similar. The expression of activation markers (IL-2, MHC Class II) on CD4, CD8 and  $\gamma\delta$ T cells is shown in Fig. 8.2-8.7. 1-2% CD4 and CD8 cells expressed IL-2R with little change in expression throughout the observation period (Fig. 8.2 & 8.3.). About 50-70% of total  $\gamma\delta$ T cells expressed IL-2R throughout this study (Fig. 8.4). A maximum of 5% MHC Class II was observed on CD4, CD8 and  $\gamma\delta$ T cells (Fig. 8.5, 8.6, 8.7).

#### **8.4.2.3 *Natural Killer (NK) like cells***

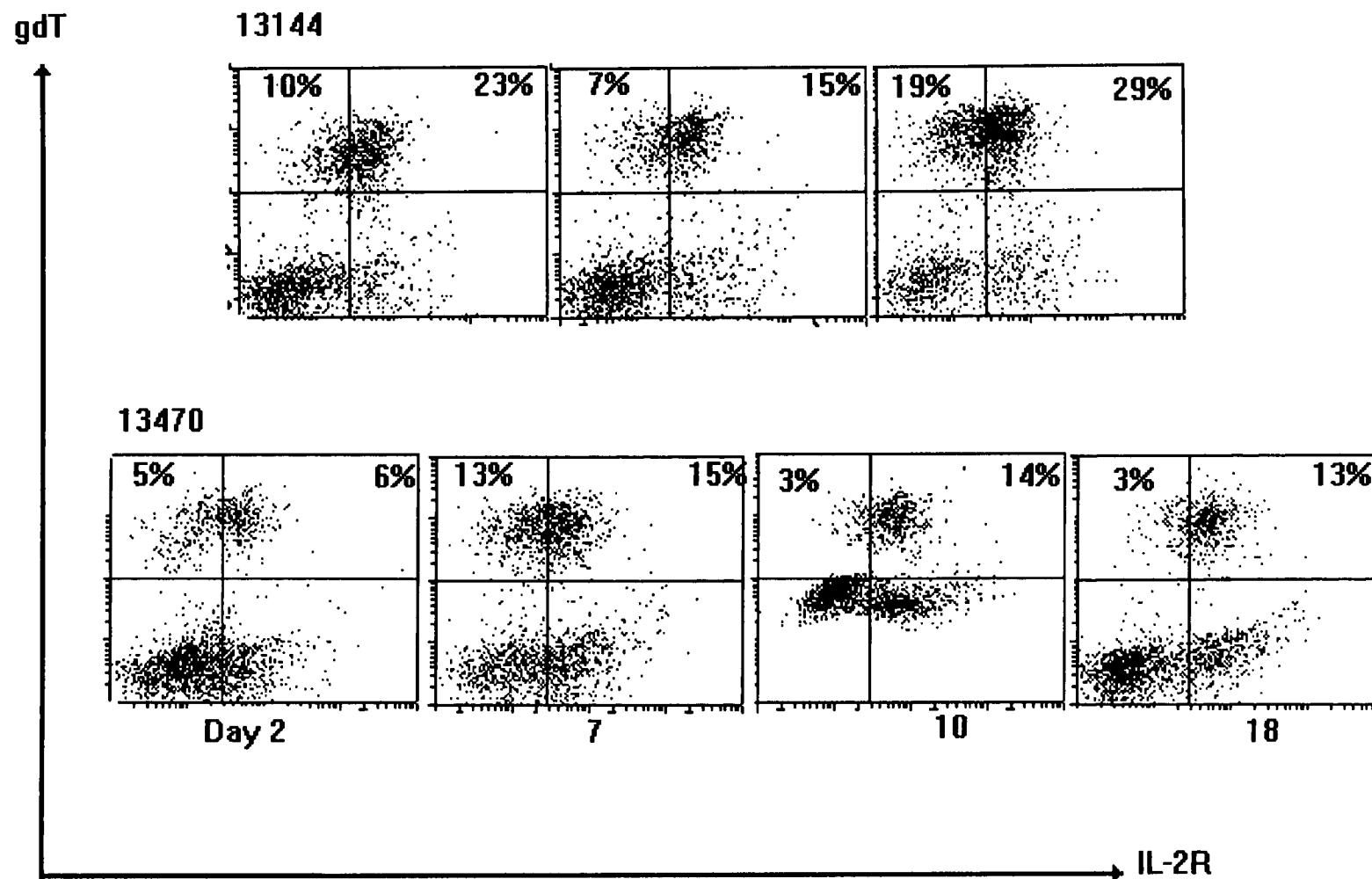


**Fig. 8.2** IL-2R expression on CD4 cells in the afferent lymph after *T. annulata* cell line immunisation. Animals: 13144, 13470

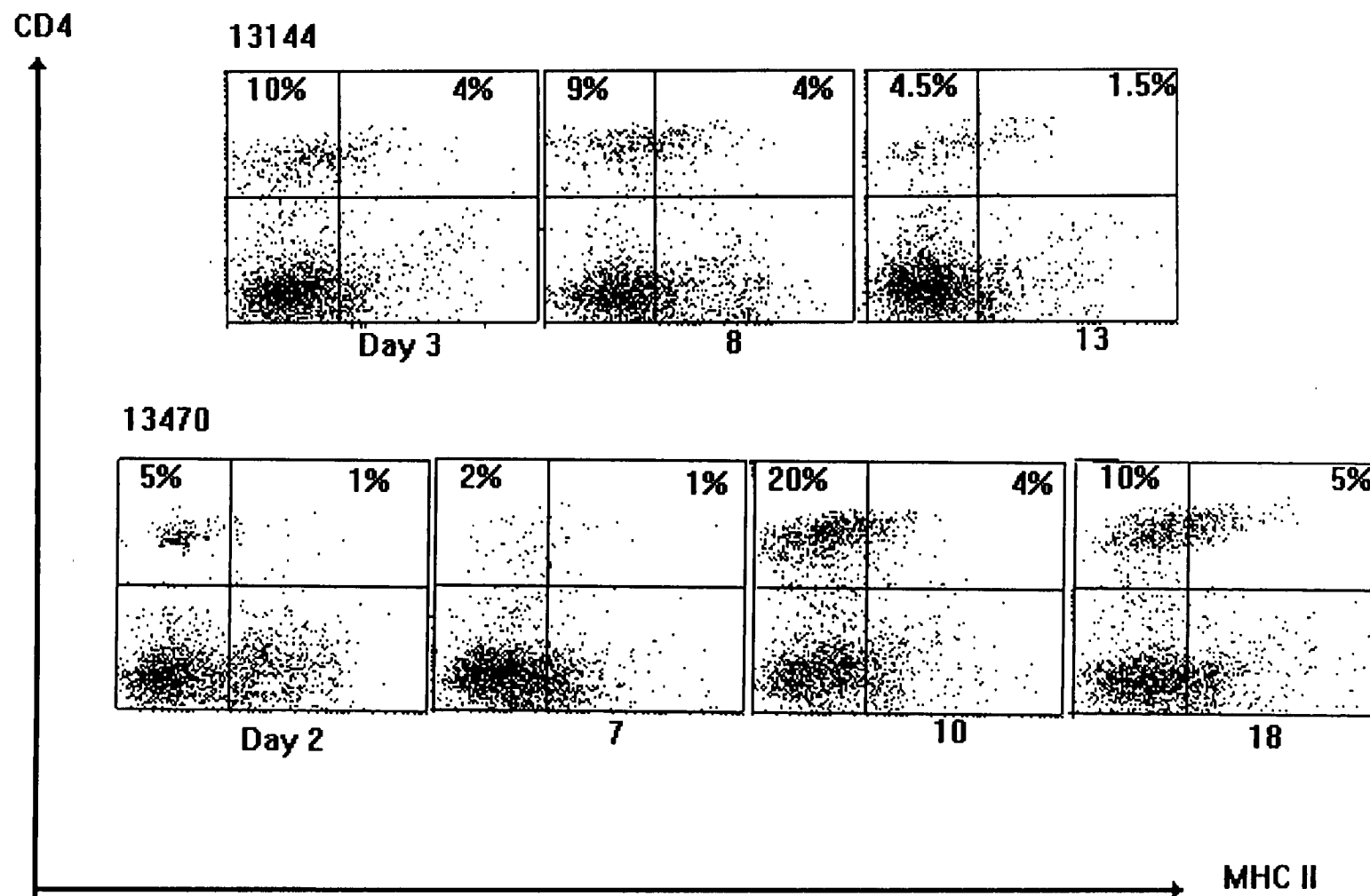


**Fig. 8.3** IL-2R expression on CD8 cells in the afferent lymph after *T. annulata* cell line immunisation. Animals: 13144, 13470



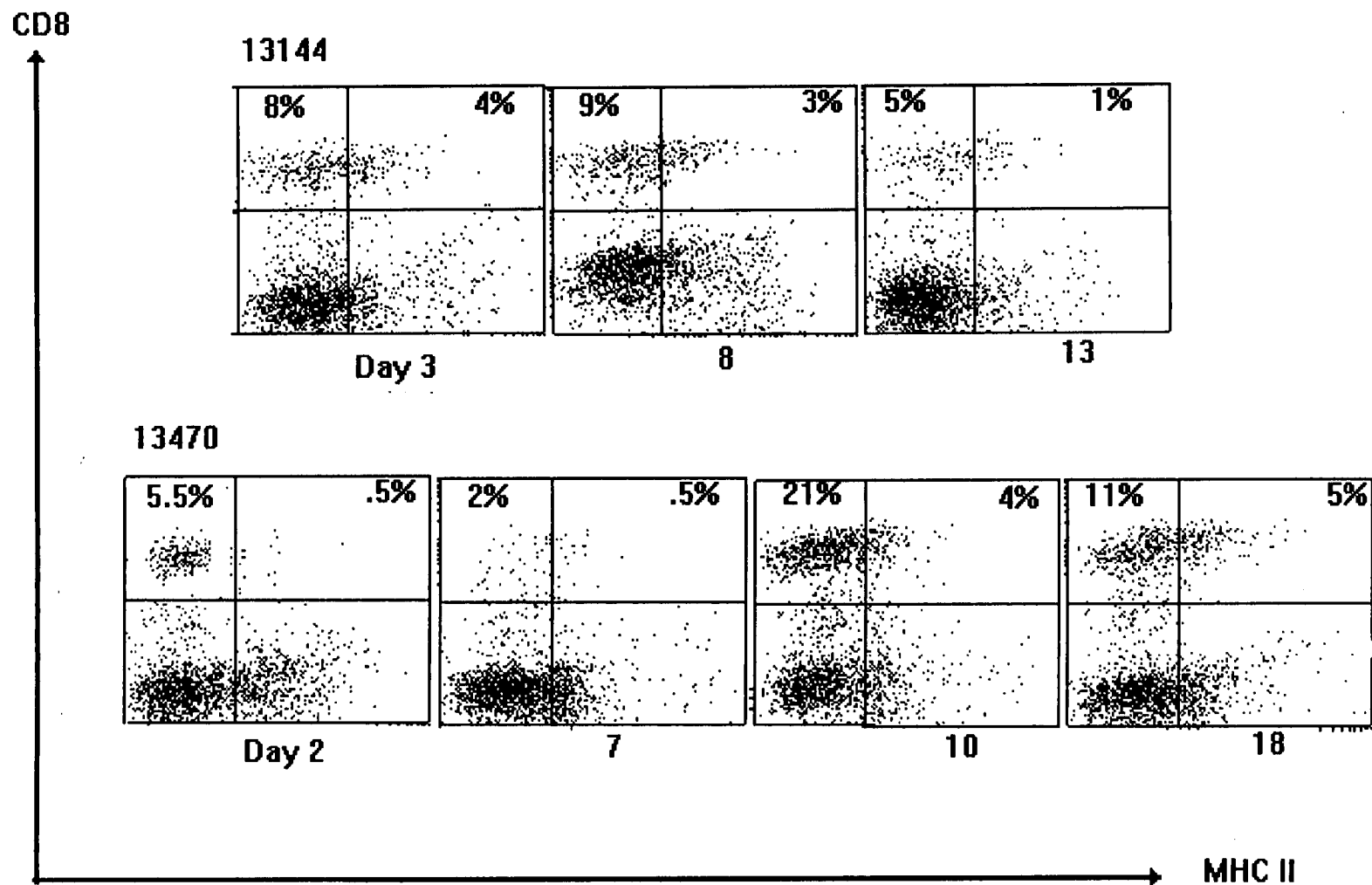


**Fig. 8.4** IL-2R expression on gdT cells in the afferent lymph after *T. annulata* cell line immunisation  
Animals: 13144, 13470

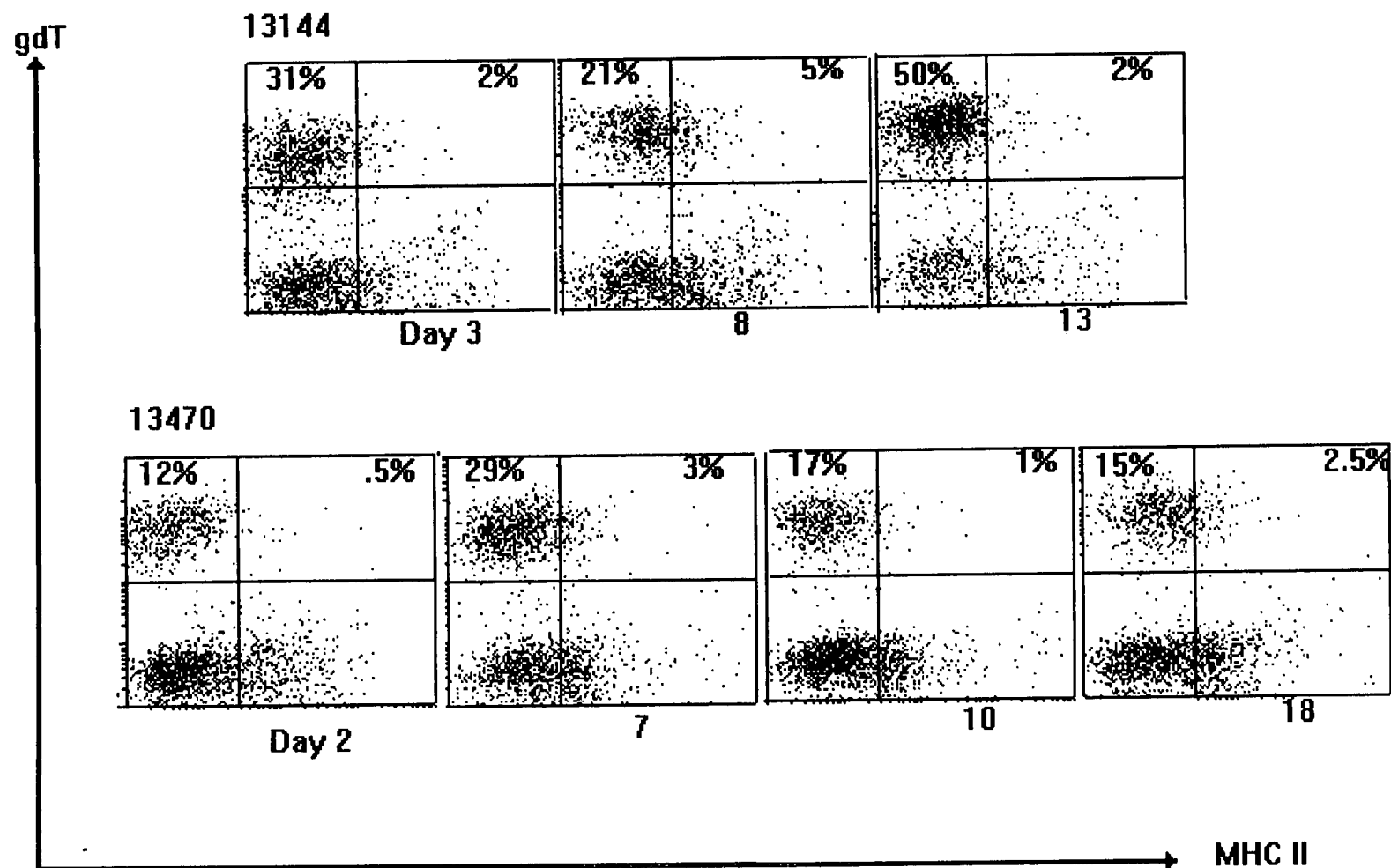


**Fig. 8.5** MHC class II expression on CD4 cells in the afferent lymph after *T. annulata* cell line immunization.

Animals: 13144, 13470



**Fig. 8.6** MHC class II expression on CD8 cells in the afferent lymph after *T. annulata* cell line immunisation  
Animals: 13144, 13470



**Fig. 8.7** MHC class II expression on gdT cells in the afferent lymph after cell line immunisation  
Animals: 13144, 13470

**Table 8.1****NK cells (Activated phenotype) (Calves: 13144, 13470)**

<b>Day /Calf</b>	<b>CD2<sup>+</sup>CD45RO<sup>+</sup></b> <b>(1) (Fig. 8.8)</b>	<b>CD4<sup>+</sup> CD45RB low +</b> <b>CD8<sup>+</sup>CD45RB low</b> <b>(2) (Fig. 8.9 &amp; 8.10)</b>	<b>CD2<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD45RO<sup>+</sup></b> <b>(NK activated type)</b> <b>( 1-2)</b>
13144			
3	21	13	7
6	23	12	11
8	23	13	10
13	16	8	8
13470			
2	4	5	0
4	17	3	14
7	18	2.5	16
10	29	20	9
14	29	13	16
18	25	9	16

CD2<sup>+</sup>CD45RO<sup>+</sup> = Memory type (activated)CD4<sup>+</sup> CD45RB low = Memory type (activated)CD8<sup>+</sup>CD45RB low = Memory type (activated)

Fig. 8.9 represents calf 13144

Fig. 8.10 represents calf 13470

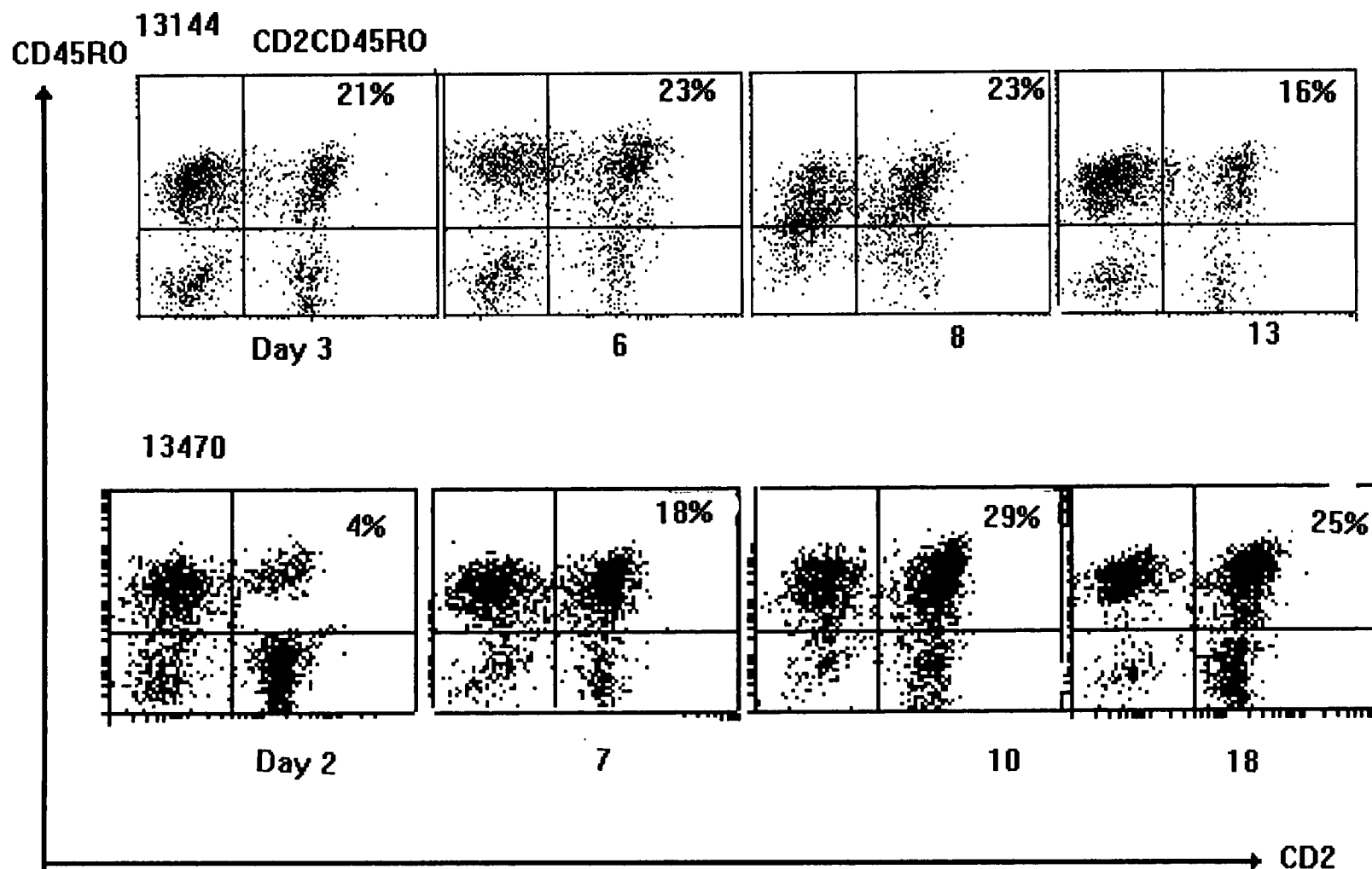
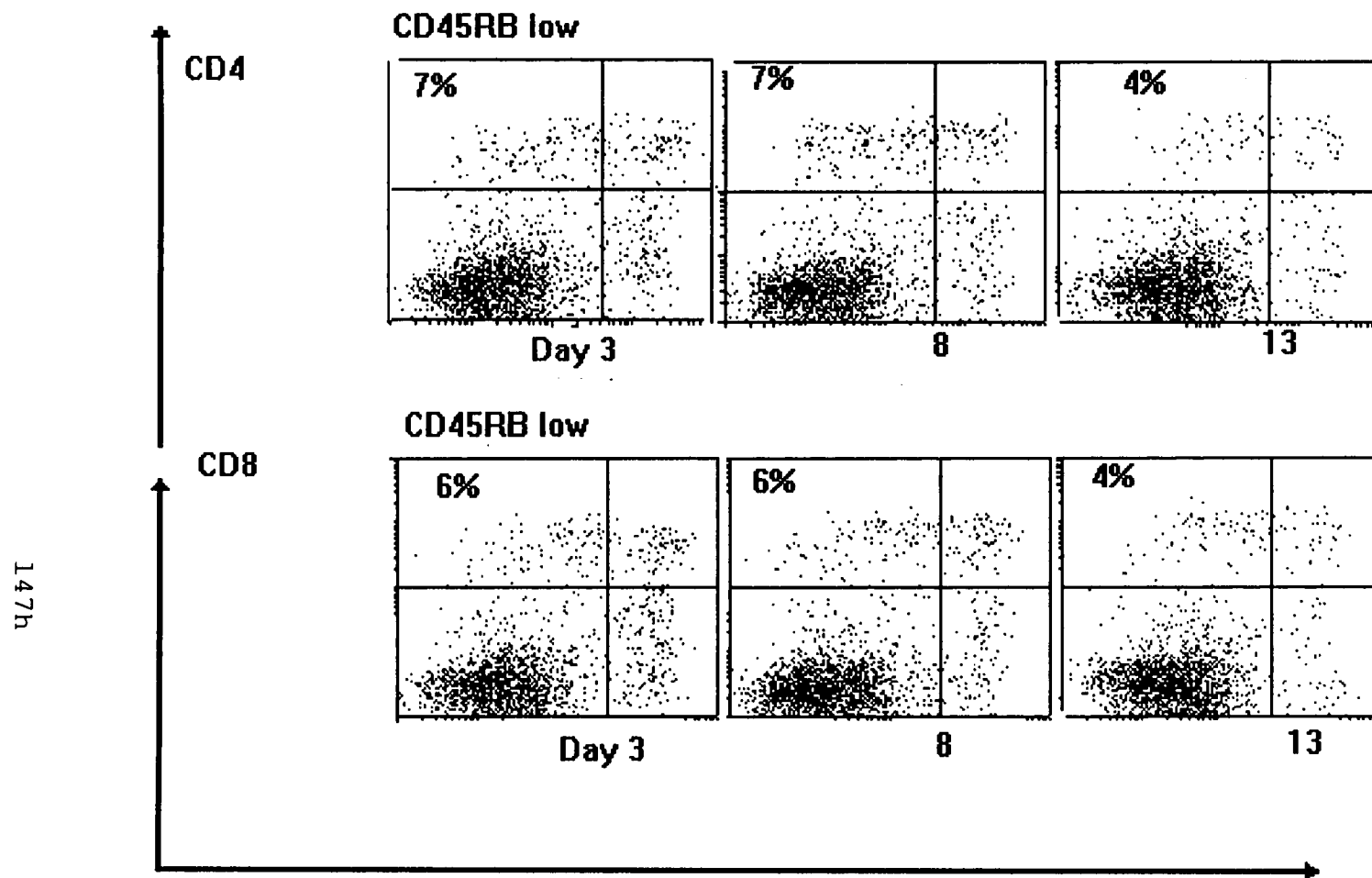


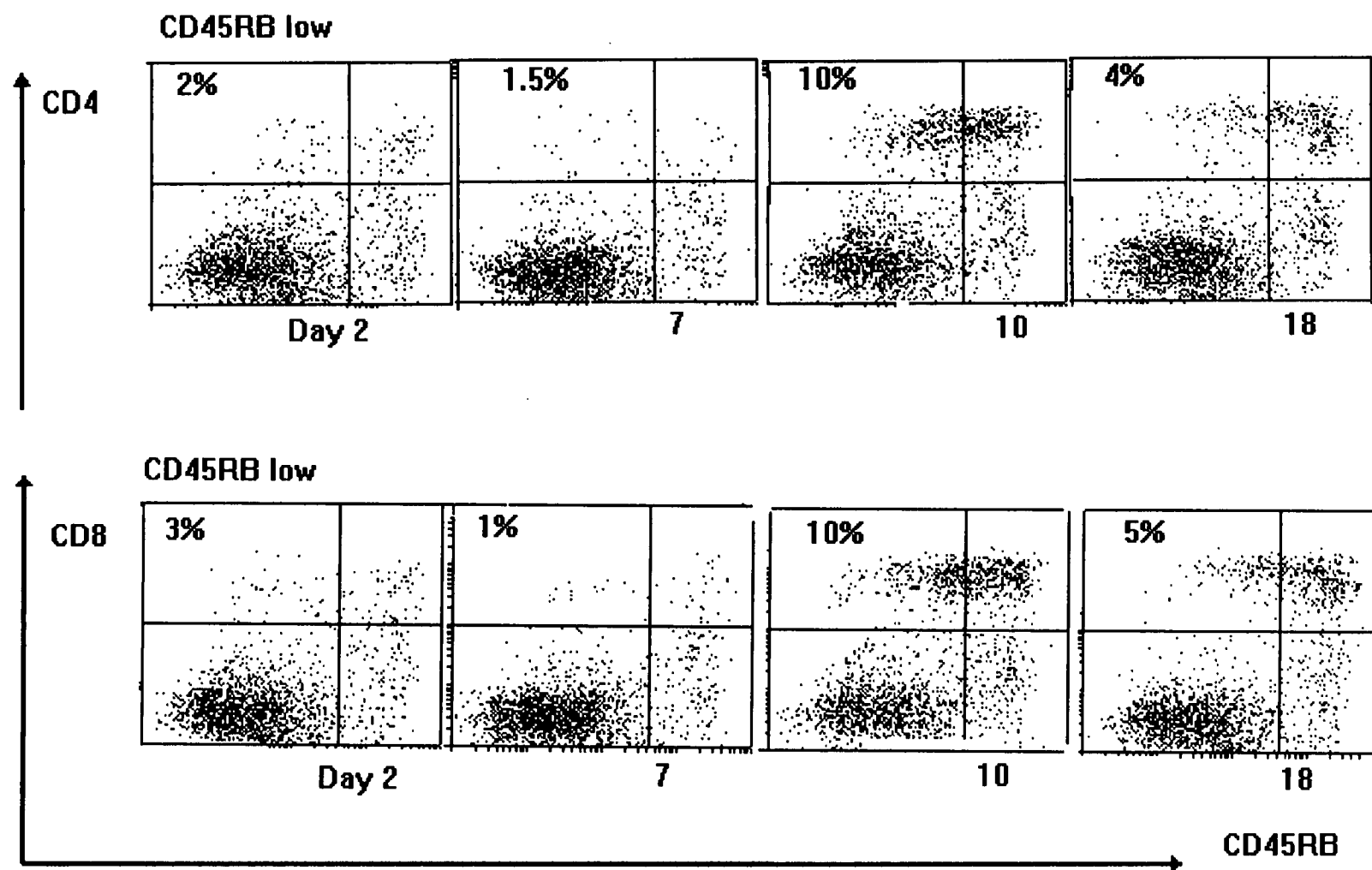
Fig. 8.8 CD45RO expression on CD2 cell in the afferent lymph after cell line immunisation

Animal: 13144

13470



**Fig. 8.9** CD45RB low expression on CD4,CD8 cells in the afferent lymph after cell line immunisation  
Animal: 13144



**Fig. 8.10** CD45RB low expression on CD4, CD8 cell in the afferent lymph after immunisation  
Animal: 13470



Bovine NK like cells are CD2<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> (Evans & Jacofriedmann 1995) and cells with the phenotype CD2<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> expressing CD45RO (memory markers) are considered as activated NK like cells (Shen *et al*, 1995; Campbell, 1995). The numbers of CD2<sup>+</sup> CD45RO<sup>+</sup> (activated NK) cells (Fig. 8.8), activated CD4<sup>+</sup> CD45RB low (memory) and activated CD8<sup>+</sup> CD45RB low (memory) in the afferent lymph are summarised in Table 8.1 and shown in Fig. 8.9- Fig.8.10. These cell numbers are calculated as described in 8.3.2

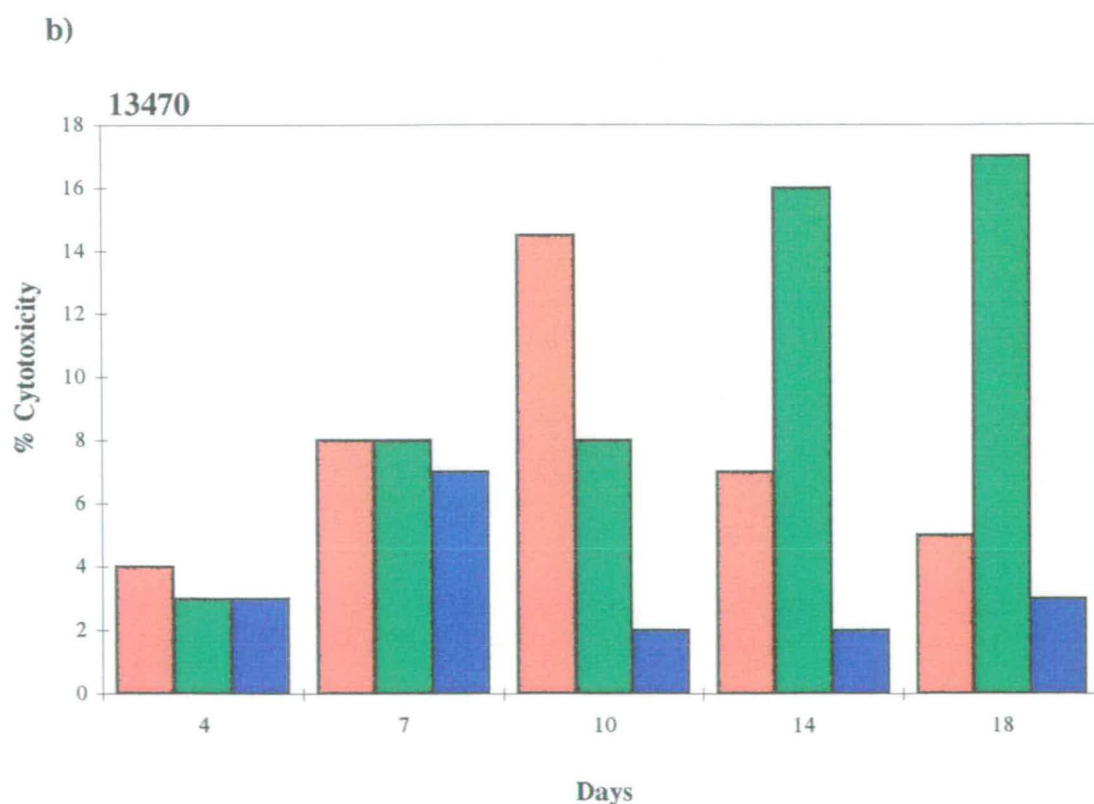
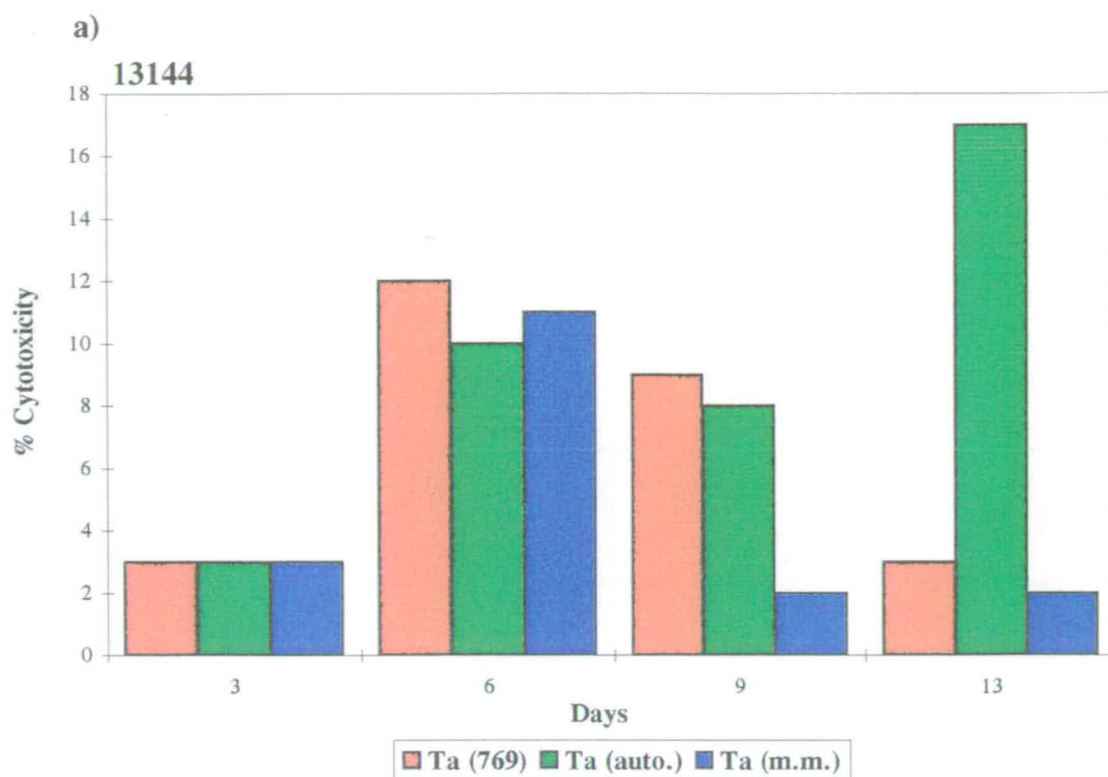
#### **8.4.3 Cytotoxic responses of afferent cells**

Cytotoxic responses are shown in Fig. 8.11. The cytotoxic activity of afferent lymph cells was assessed in a 4 hour <sup>51</sup>chromium release assay. ALC collected overnight were tested as effectors against a *T.annulata* infected mismatched cell line (m.m.), the immunising cell line (Ta 769) and an autologous cell line (Ta auto.)

In calf 13144, a 3% cytotoxicity was observed on day 3 against all the targets which increased to 11% against the mismatched cell line, 12% against the immunising cell line (Ta769) and 10% against the autologous cell line (Ta auto), by day 6. Whereas the percent cytotoxicity against the mismatched cell line decreased to 2% after day 6, it increased against other two types of targets cells (Fig. 8.11). A similar pattern of cytotoxicity was observed with the ALC of calf 13470 (Fig. 8.11).

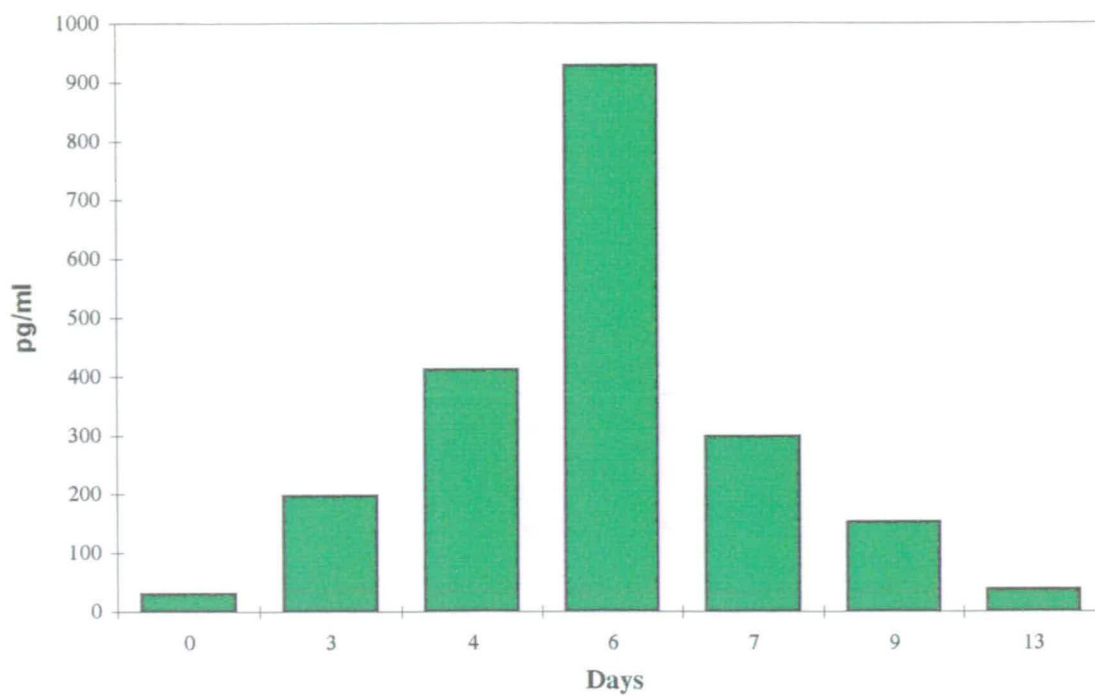
#### **8.4.4 Interferon $\gamma$ in the afferent lymph plasma**

The levels of IFN $\gamma$  detected in the afferent lymph after immunisation are shown in Fig. 8.12. IFN $\gamma$  was 30pg/ml on day 0 but reached a maximum of 30 times higher (900pg/ml) during the first week after immunisation. Lymph on day 0 was collected from the calves 12886 & 13130 (used in last chapter) as it was

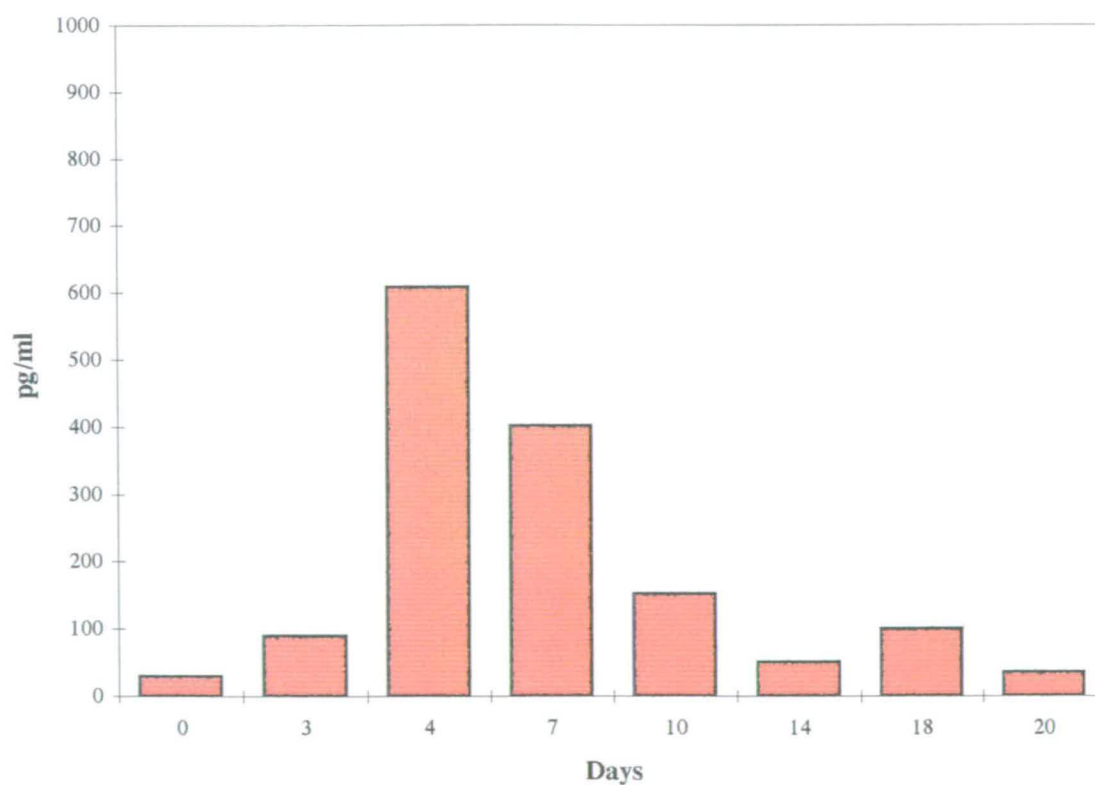


**Fig. 8.11** Cytotoxic responses of afferent lymph cells (ALC) of a) calf 13144  
b) calf 13470 incubated with *T. annulata* immunising (Ta 769),  
*T. annulata* autologous (Ta auto.) and *T. annulata* mismatch (Ta  
m.m.) cell lines in a 4 hour chromium release assay  
Results expressed as percent (%) cytotoxicity by ALC.

a)



b)



**Fig. 8.12** Interferon gamma (pico gram / ml) in the afferent lymph plasma of  
a) calf 13144 b) calf 13470 following immunisation with *T. annulata*  
(10769) cell line.

Day 0 represents lymph collected before immunisation.

not possible to collect the day 0 lymph in calves 13144 & 13470.

## **8.5 DISCUSSION**

During this study activated NK like cells, IFN $\gamma$  and non-MHC restricted cytotoxicity was recorded in the afferent lymph after immunisation. Most of this discussion therefore deals with the NK like cells in relation to IFN $\gamma$  and non-MHC restricted cytotoxicity. Although IL-12 could not be assessed it has been discussed along with IFN $\gamma$  and NK due to some recent interesting reports about its role.

The clinical observations were almost the same as were observed in chapter 6. The appearance of piroplasms at day 7 in one of the animals was unexpected. Normally they appear in the 2nd week (12-18 days) observed in chapter 3, 6. In this chapter, animals were first immunised close to the position of an extirpated lymph node and then cannulated i.e. the cell line was allowed to enter the circulation. The cell line then entered the circulation immediately which was much earlier than when draining through a lymph node where the earliest time appeared to 12 days after immunisation (Nichani, 1995).

No great difference was observed in the activation pattern of T cells as compared to the last chapter, except that the number of CD8 cells was high from the first day of observation. This could be due to the recruitment of more CD8 cells to the site of immunisation in response to the cell line already in the circulation before cannulation.

### **8.5.1 Natural killer like activity**

In the last chapter, afferent lymph cells exhibiting a non-MHC restricted cytotoxicity were taken to be natural killer cells. This cytotoxicity was thought to be due to NK

cells as cells with NK cell phenotype ( $CD2^+CD4^-CD8^-$ ) were present in the lymph. In this experiment,  $CD2^+CD4^-CD8^-CD45RO^+$  cells were observed in afferent lymph after immunisation with a cell line. As explained above (Table 8.1) these cells are thought to have been activated NK cells. These observations raise the question as to the nature of the mechanism involved in activation of  $CD2^+CD4^-CD8^-$  (NK) cells.

In contrast to the human and mouse systems, which have significant endogenous NK activity and which when treated with cytokines increase their endogenous activity (Lattime *et al*, 1986; Ortaldo *et al*, 1986), cattle NK cells have little endogenous activity prior to activation by cytokines (Jenson & Schultz 1990). Similar findings were observed in this study. In man, IL-2, IFN $\alpha$  and IFN $\gamma$  have been proposed to be responsible for activation. IL-2 activates indirectly through IFN $\gamma$  whereas IFN $\gamma$  has its direct effect (Henney *et al*, 1981; Weigert *et al*, 1983; Djeu *et al*, 1979; Senik *et al*, 1979). NK cells have been proposed to be most powerful producer of IFN $\gamma$  (Scott & Trinchieri, 1995). In this study IFN $\gamma$  was shown to be present in the afferent lymph collected during the first 10 days after immunisation at a time when cells with activated NK cells phenotype were present (Fig. 8.12). During this period ALC were shown to kill *T. annulata* infected cells in a non-MHC restricted manner indicating NK cell activity (Fig. 8.11).

IL-12 has been shown to play a central role in the regulation of NK activity in other situations (Kobayashi *et al*, 1989; Gately *et al*, 1991; Chan *et al*, 1991; D'Andrea *et al*, 1992). Phagocytic cells i.e. monocytes, macrophages and neutrophils produce IL-12 in response to bacteria and intracellular parasites (Kobayashi *et al*, 1989; D'Andrea *et al*, 1992; Gazzinelli *et al*, 1993). The main targets for IL-12 have been suggested to be T and NK cells and major direct biological function of IL-12 on these

cells is thought to be the induction of cytokine production, primarily IFN $\gamma$ , and the augmentation of cytotoxicity (Kobayashi *et al*, 1989). Recently, the same findings have been reported for increased potent non-MHC restricted cytotoxicity for liver NK cells in mice (Hashimoto *et al*, 1995; Anzai *et al*, 1996). Although IL-12 production has not been studied in *T. annulata* infection it seems likely that this cytokine will play a similar role in regulation of bovine NK cells.

*Corynebacterium parvum* has been used as an immunostimulant alone or as an adjuvant with live or dead vaccines to enhance the resistance of the animals to *Plasmodium* spp (Nussenzweig, 1967), *Trypanosoma cruzi* (Bomford & McHardy, 1979), *Babesia* spp (Clark *et al*, 1977) *Toxoplasma gondii* (Swartzsberz *et al* 1975). Although attempts to increase resistance to *Theileria* with the use of parasite extracts and adjuvants such as Freund's, Saponin or with BCG were unsuccessful (Wilde *et al*, 1968; Wagner *et al*, 1974; Pipano *et al* 1977 and Dolan *et al*, 1980), an attempt using *C. parvum* was successful (Manickam *et al*, 1983). Although no attempts were made to identify the mechanism of action in this instance, it is very likely that *C. parvum* had stimulated the NK cells and enhanced the nonspecific immunity in the immunised animals.

In situations where NK cells are missing, life threatening situations have been reported e.g. in relapsing virus infection in humans (Biron *et al*, 1989). It has recently been shown that CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup> NK cells are essential for the induction of CTL and differentiation of CD8 into effector CTL in the presence of NK requires direct contact between CD8 and NK (Kos & Engleman, 1995). Taken together the above studies suggest that a dynamic balance between NK and CTL system is essential to defence against microbial infections including those initiated with *T. annulata*.

In summary, although the number of animals was small the presence in the afferent lymph of activated NK cells with a non MHC restricted cytotoxicity and IFN $\gamma$  suggest the nonspecific natural immune mechanism involving NK cells as the first line of defence (at least at the site of immunisation). The MHC restricted CTL were active from day 10-18 after immunisation (Fig. 8.11) suggesting that these cells act as an effector mechanism later on.

## **CHAPTER 9**

### **SUMMARY AND CONCLUSIONS**



## Introduction

This is the first description of immune responses in the DLN and afferent lymph stimulated in cattle by inoculating with *T. annulata* macroschizont-infected cell lines. Inoculation with allogeneic cell lines stimulated only a mild clinical reaction as monitored by pyrexia and detected by slight fall in haematocrit and circulating leucocytes. Both macroschizonts and piroplasms were observed during the third week after inoculation. Challenge of two cattle inoculated with cell lines showed that this immunising regime had protected the animals against infections with sporozoites. The clinical response and resistance to challenge described here resembled those obtained previously using allogeneic cell lines (Innes *et al*, 1989a).

In brief, DLN studies showed the formation of GC and T cell activation following cell line immunisation. RT-PCR studies on DLN cells showed the production of IL-2, IL-2R, IFN $\gamma$  followed by IL-4. Cannulation studies indicated the DLN of cattle to be the site of parasite transfer and showed: the circulation of naive T cells through the afferent lymphatics, the site of immunisation as the initial T cell activation site and NK-like activity at the site of immunisation. Together these results provided evidence that NK cells act as an initial protective mechanism against inoculation with cell lines followed later on by CTL.

### 9.1 Germinal centre formation and T cell activation in the draining lymph node

Well developed GCs were recorded by day 16 after immunisation. The GCs are the specialised area of the lymph node where T cell dependent B cell maturation is believed to occur. The B cells proliferate extensively in the GC (Fliedner *et al*, 1964) and the T cells are believed to be antigen specific in the GC (Kathleen *et al*, 1993). Upon immunohistological examination, activated T (IL-2R<sup>+</sup>) cells were observed

initially at their normal anatomical sites in and around HEV and in the paracortex. Similar findings were first reported by Bogen *et al*, (1991), when they found IL-2<sup>+</sup> cells by day 4 after protein antigen. After the IL-2R<sup>+</sup> cells appeared in the paracortex in this study, the number of T cells increased in the B cell area. Presence of T cells in the primary follicle of B cell area is believed to be critical for the GC formation as the GC do not form without T cell help (Jacobson *et al*, 1974). In this study, T cells were rare in the PF of the normal node but their numbers gradually increased in the PF of immunised calves as the period after immunisation increased i.e. there were a least 15-20 T cells in each of the well developed GC by day 16 of immunisation.

The activation state of T cells was revealed in *in vitro* assays in which the DLN cells proliferated initially in response to MHC antigens of the immunising cell lines and later to the autologous parasite infected cell lines. The DLN cells also proliferated in response to exogenous IL-2. Results indicated that the DLN was responding to immunisation with cell lines as reported for protein antigens (Bogen *et al*, 1991).

Cytokine studies were performed to show the cytokine profiles of the DLN cells after immunisation. The loss of mRNA for IL-4 and an increase in mRNA for IFN $\gamma$  were observed by day 4. Both of these mRNAs were undetectable by day 12 but reappeared on day 16. This is in complete contrast to the cytokine profile seen in lethal infections induced by sporozoite (Campbell, 1995). Presence of IL-2 and IL-2R indicated the occurrence of activated T cells in the DLN.

Early IFN $\gamma$  production during DLN responses has been suggested to influence subsequent T cell activation (Bogen *et al*, 1993). IFN $\gamma$  is a strong antagonist to IL-4

producing cells (Donckier *et al*, 1994). Observations in the literature strongly suggest the production of IFN $\gamma$  is controlled by IL-4 thereby preventing its direct inflammatory effect (Roberts *et al*, 1996). It seemed as though a similar mechanism was in operation early after cell line immunisation. A strong mRNA response for IFN $\gamma$  was accompanied by the loss of IL-4 on day 4. However by day 9, the mRNA band for IFN $\gamma$  was weak and lacking altogether by day 12. The interplay of IL-4 and IFN $\gamma$  is crucial for the outcome of any infection or immunisation. This may also be true in *T. annulata* infections. In acute theileriosis, the early production of the IFN $\gamma$  was accompanied with the total loss of IL-4 production whereas in immunised animals undergoing selfhealing infection, IL-4 occurred together with IFN $\gamma$ . The production of IL-4 late in immunisation might reflect a dynamic *in vivo* balance, preventing an overexpression of inflammatory, potentially tissue damaging, IFN $\gamma$ .

The T cell stimulates B cell to proliferate (dark zone) and IL-4 switches these B cells to antibody diversification (light zone). The final selection of the B cells takes place in the light zone, when the B cells either become functional or die by apoptosis (MacLennan, 1994). In sporozoite infection the majority of B cells are being prevented from entering the light zone, possibly by IFN $\gamma$  (Campbell, 1995) whereas VPM30<sup>+</sup> B cells were observed in this study possibly due to the reappearance of IL-4.

The weak band of IL-4 shown by RT-PCR on day 16 was unexpected as hyperplastic GC with peak antibody titers were recorded on day 16 after immunisation. This may have been due to downregulation by IFN $\gamma$ .

To summarise, sequential production of IFN $\gamma$  and IL-4 seemed to be delicately balanced as inappropriate or excessive production of either could have caused

pathology in the DLN.

## **9.2 Activation of T cell and NK cells in the afferent lymph**

After completing the studies on the DLN, investigations into the immune responses in the afferent lymph were undertaken by cannulating the pseudoafferent duct. These studies were performed to examine the activity of T cells & NK like cells and to identify the site for parasite transfer. There were no previous reports regarding the isolation of infected cells of donor origin and no experiment had been undertaken before to establish the site of parasite transfer.

A novel method for collecting lymph was devised and proved successful without putting the animal under unnecessary stress. Animals were immunised with *T. annulata* infected cells stained with a fluorescent dye to locate the site of parasite transfer. PKH2 stained cells of the immunising cell line drained into the collecting bottle within minutes of injection. It is interesting that large number of cells found their way into the lymph so quickly. The *T. annulata* infected cell lines reisolated from the afferent lymph immediately after cannulation were also of donor origin. This was confirmed by BoLA typing of the reisolated cell lines. This study has shown for the first time that *T. annulata* infected cells of the immunising cell line drained immediately to the draining lymph node after immunisation. Since cells of donor origin were not seen in efferent lymph and the first infected cells of recipient origin were not seen before day 12 (Nichani, 1995), it seems likely that the infected donor cells must have been residing in the node during this time and that parasite transfer from donor to recipients cells took place in the node. Afferent lymphatics were the main route of parasite dissemination from the site of immunisation to the draining lymph node. All the cells drained through the afferent lymphatic system were

collected and removed in the bottle. Even then the presence of parasite in the blood of all the animals indicated that some of the cells took some other route after immunisation.

The T cell activation in the afferent lymph was examined by flow cytometry. An increase in CD2 was associated with an increase in CD8 and a decrease in  $\gamma\delta$  T cells. There was marked increase in the expression of MHC Class II with a little increase in IL-2R on CD8 cells after immunisation. Cytotoxic assays revealed that ALC were cytotoxic for allogeneic cells of the immunising cell line and for the autologous cell line. At the same time proliferative responses of the ALC increased markedly in response to incubation with *T. annulata* infected cells belonging to the immunising cell line and to the *T. annulata* infected autologous cells. These functional assays showed that the lymphocytes coming out of the afferent lymph were already committed either to antigen of the immunising cell line or to those of the autologous parasite infected cell line and were therefore acting as memory cells.

The majority of CD4 cells expressed CD45RB low (memory/effector type) in the resting afferent lymph. This was found throughout the observation period after immunisation except for a slight increase in the CD45RB high during the late stage of immunisation. Increase in CD8 cells was associated with the CD45RB high (naive) population. The presence of CD45RB high (naive) cells in the afferent lymph showed that even naive T cells could circulate through the skin in to the afferent lymph. Until 1995, it was believed that only the memory cells circulate through the skin but in a single report it has been shown that naive T cells can also circulate through the skin (Washington *et al*, 1995). Findings in this thesis are in complete accord to that of Washington's study. There are no other reports to show that naive T cells can also

circulate through the skin.

Activated NK like cells (CD2<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD45RO<sup>+</sup>) and IFN $\gamma$  were observed in the afferent lymph after immunisation. It seems likely that the NK cells were activated and expressed the activated phenotype (CD45RO<sup>+</sup>) in the surrounding IFN $\gamma$  rich environment. Further confirmation of their activated state was obtained by the killing of *T.annulata* infected cells in a non-MHC restricted manner.

In summary, these studies showed that cattle inoculated with cell lines expressed a competent immune response in the DLN, involving IL-2, IL-2R, IFN $\gamma$  and IL-4, and that the node itself appeared to be the site where the parasite transferred from the donor to the recipient's cells. Both the initial T cell activation and NK cell activity were found to occur at the site of immunisation suggesting a protective role for NK cells in selfhealing infections initiated by inoculation with *T. annulata* infected cell lines.

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## APPENDIX

### MHC class I typing

#### 1.1 Hanks balanced salt solution

The HBBS was supplied in powder form (Gibco BRL) containing phenol red and was reconstituted with deionised distilled water. Sodium bicarbonate was added at the rate of 0.35g per liter and the pH of the HBBS solution was adjusted to 6.8 with a pH meter using 1N NaOH. The final volume was made up to 100 ml to make it 10 times concentrate (10x). It was sterilised by filtration (0.22 $\mu$ , Millipore Corporation) and stored at 4°C until required. The solution was made 1x just before use with the addition of sterilised distilled water.

#### 1.2 Complement

Complement was obtained from the pooled rabbit sera collected from a commercial slaughterer. Batches of complement were screened for lack of inherent cytotoxicity and for potency in a standard lymphocytotoxicity test using test cells and antisera of known reactivity. Suitable batches of complement were then stored at -70°C until required.

#### 1.3 Eosin dye

5% w/v Eosin powder (Koch Light Laboratories) in HBBS.

#### 1.4 Fixing solution

5ml (0.15M) disodium hydrogen phosphate in deionised distilled water, 95ml formalin.

#### 1.5 Terasaki typing plates

Terasaki 60 well plastic plates (Intermed, Nunc), 1 $\mu$ l typing sera (per well) covered by paraffin using a multidispenser Hamilton syringe.

#### 1.6 ACD

2.5% D-Glucose (Fisons) and 2.05% Di-Sodium Hydrogen Citrate (Fisons) w/v aqueous solution. Used as 1ml for 4 ml blood.

### 2 Tissue culture

#### 2.1 Ammonium chloride lysis solution

0.16M Ammonium Chloride (Fisons), 0.17M Tris (Fisons) mixed 9:1 just before use.

#### 2.2 PBS

0.9% Sodium Chloride, 0.107% Di-Sodium Hydrogen Phosphate, 0.051% Sodium Di-Hydrogen Orthophosphate (all Fisons) w/v aqueous solution pH 7.2

#### 2.3 Complete Tissue culture medium

RPMI 1640 medium containing 25mM Hepes, supplemented with 2mM glutamine, 100 IU/ml penicillin, 100 $\mu$ g/ml streptomycin (all Gibco BRL), 10% FCS (Meridien).

#### 2.4 Mixed lymphocyte culture medium

RPMI 1640 with 25mM Hepes supplemented with 10% FCS, 2mM L-Glutamine, 100 $\mu$ g/ml Gentamycin (Gibco) and 5x10<sup>-5</sup>M  $\beta$ -Mercaptoethanol (Sigma).

### 3 PCR studies

### **3.1 Loading buffer**

50% v/v glycerol (Fisons), 1mM EDTA (Sigma), 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF.

### **3.2 PCR buffer (Gibco)**

200mM tris-HCl pH 8.4, 500mM KCl

### **3.3 dNTPs (Pharmacia)**

100mM stock diluted to 10mM in TE buffer.

### **3.4 TE**

10mM Tris-HCl pH 7.6, 1mM EDTA

### **3.5 TBE**

10x stock solution: 108gm Tris, 55gm boric acid, 40ml 0.5M EDTA (pH 8) in 1 liter dist. water,. Diluted to 1x before use.

### **3.6 HaeIII markers**

Hae III digest of PBR 322 plasmid (Sigma)

## **4 LYMPH NODE**

### **4.1 Eosin solution**

1% w/v Eosin and 1% w/v Phloxine (BDH) aqueous solution.

### **4.2 TBS**

0.9% NaCl buffered with 50mM Trisma base (Sigma) pH 7.6 with 1N HCl.

### **4.3 Trypsin Digestion solution**

0.1% w/v Trypsin 1:250 (DIFCO, Detroit, USA) and 0.1%  $\text{CaCl}_2$  (BDH, Poole, UK) in distilled water.

### **4.4 Pronase Digestion solution**

75mg/ 100ml pronase (Sigma) in TBS= 4.1 U/ml

### **4.5 Citric Acid Solution**

1.05gm citric acid (BDH) in 500ml distilled water, pH 6.

### **4.6 DAB substrate Buffer**

50mM Tris-HCl pH 7.6 plus 0.01M Imadizol (Sigma). 5mg DAB and 1 drop 30 v  $\text{H}_2\text{O}_2$  to 5ml buffer just before use.

### **4.7 Pepsin Digestion Solution**

0.5% pepsin (Sigma) in 0.9% NaCl solution pH 6.

### **4.8 Propidium Iodide Solution**

100 $\mu\text{g}/\text{ml}$  aqueous solution PI (Sigma) with 0.04% RNase. (Sigma RNase A from bovine pancreas)



## LIST OF PUBLICATIONS FROM THE THESIS

1. **P Goel, EJ Glass, PM Preston & RL Spooner.** (1995). Kinetics of phagocytes and lymphocytes subpopulations in the afferent lymph of cattle after *Theileria annulata* cell line immunisation. Presented at "Phagocyte Discussion Meeting", Medical School, Edinburgh. 15th September, 1995.
2. **P Goel, JDM Campbell, BH Thorp, PM Preston & RL Spooner.** (1995). Immune responses in the draining lymph node after *Theileria annulata* cell line immunisation in cattle. Immunology, 86- (1).
3. **John DM Campbell, Sarah EM Howie, John Hopkins, Robert A Oliver, Parveen Goel, Roger L Spooner & Elizabeth J Glass.** (1995). A novel cell surface proliferation-associated marker expressed on T cells and Germinal Centre B cells. Immunology, 86- (1).
4. **P Goel, J.D.M. Campbell, E.J. Glass, P.M. Preston & R.L. Spooner.** (1996). T cell activation in the afferent lymph after *Theileria annulata* cell line immunisation. In: Proceedings of 6th International conference on Lymphocyte activation and Immune regulation. 2-4th Feb, 1996, held at Newport Beach, California, USA.
5. **P Goel, M Ritchie, PM Preston & RL Spooner.** (1996). Responses of the draining lymph node to a primary cell line immunisation in bovine tropical theileriosis. In: Proceedings of 14th International symposium of the World Association of Veterinary Microbiologist, Immunologist and specialist in infectious diseases. 3-5 July, 1996. UK.
6. **P Goel, JDM Campbell, DV Malhotra, PM Preston & RL Spooner.** (1996). Clinical observations in *Theileria annulata* infected cell line immunised and challenged calves in the absence of draining lymph node. In: Proceedings of XIX World Buiatrics Congress. 8-12th July, 1996. Edinburgh.UK

Vol 86, Supplement 1, December 1995

# Immunology



## OP151 IMMUNE RESPONSES IN THE DRAINING LYMPH NODE AFTER *THEILERIA ANNULATA* CELL LINE IMMUNISATION IN CATTLE.

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Following *Theileria annulata* sporozoite infection, there is a large burden of parasite in the draining lymph node (DLN, initial site for parasite development), disruption in T cell activation, destruction of existing germinal centers (GC). In order to compare this apparent immune response failure with a protective response, we have studied the immune responses in the DLN after cell line immunisation. Activated T cells (IL-2R<sup>+</sup>) were observed near high endothelial venules (anatomical sites for normal activation) in the paracortex and in the GC. Large numbers of GC with dark and light zones developed by day 16 of immunisation. T cells were observed in the paracortex and in the T dependent zone of GC. Differentiated B cells were observed primarily in the light zones of GC. These results will be discussed in relation to the failure of immune response in sporozoite infection.

## IMMUNOLOGY (86) SUPPLEMENT 1

### OP208 A NOVEL CELL SURFACE PROLIFERATION-ASSOCIATED MARKER EXPRESSED ON T CELLS AND GERMINAL CENTRE B CELLS

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We report here the identification of a novel ruminant lymphocyte cell surface antigen (Ag), mwt. 28kD (reduced) recognised by mAb VPM30. Originally thought to be restricted to peripheral B cells, the results here show a much wider cellular distribution of the Ag and a strong association between cell proliferation and Ag expression. CD4<sup>+</sup>, CD8<sup>+</sup> and  $\gamma\delta$ T cells rapidly express the Ag upon Con A activation, with expression waning as proliferation decreases. Inhibition of cell division completely blocks Ag expression on T cells. In paraffin sections of lymph nodes the Ag is only found on B cells in the light zone (LZ) of germinal centres (GC), not in the dark zone. There is strong evidence to suggest that the expression of the Ag is dependant upon "rescue" of B cells in the LZ. Others have shown that such rescue is dependant upon expression of Ig by B cells, and that Ig expression is inhibited by the action of IFN $\gamma$ . In a parasite infection associated with over production of IFN $\gamma$  within LN, currently under examination in our lab, expression of the novel Ag was shown to be completely blocked in GC.

# T CELL ACTIVATION IN THE AFFERENT LYMPH AFTER *THEILERIA ANNULATA* CELL LINE IMMUNISATION

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## INTRODUCTION

The protozoan parasite, *Theileria annulata*, causes a lymphoproliferative disease of cattle known as tropical theileriosis. *T. annulata* infected *in vitro* cell lines successfully immunise and protect cattle and are used as vaccines. Immune responses to *T. annulata* infected cell lines develop in the draining lymph node (Goel, 1994) and in the efferent lymph (Nichani, 1994). Activated CD8<sup>+</sup> T cells exit in the lymph draining the node. These activated CD8<sup>+</sup> T cells in the efferent lymph (Nichani, 1994) and in the peripheral blood are cytotoxic to the allogeneic immunising cell line and to the parasite (Innes et al., 1989; Nichani, 1994). Reisolated cell lines from the PBM and efferent lymph cells of immunised animals were reported to be of recipient animals. The transfer of parasite from the immunising cells to the cells of recipient is considered to be essential for immunity (Brown et al., 1978; Innes et al., 1989; Nichani, 1994). Bovine afferent lymph contains about 50% T cells (Emery et al., 1987). However their level of activation and immunocompetence is unknown and their role in dissemination of an immune response is unclear.

## OBJECTIVES

- 1) To investigate the site of parasite transfer, development and dissemination in the afferent lymph draining the site of immunisation with an allogeneic *T. annulata* infected cell line.
- 2) To study the dynamics of various lymphocyte subpopulations in the afferent lymph draining the site of immunisation in relation to the development and systemic dissemination of an immune response.
- 3) To characterise the activation of afferent lymph T cells draining the site of immunisation with an allogeneic *T. annulata* infected cell line.
- 4) To investigate the immune status of the immunised animals in the absence of draining lymph node.

To achieve these objectives, calves were cannulated prior to inoculation of a cell line and lymph was collected for various assays.

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 Goel P. (1994). Second year Ph.D report. University of Edinburgh. Edinburgh. UK.  
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## MATERIAL AND METHODS

- 1) Prescapular lymph nodes were removed to allow afferent lymphatics to anastomose with the efferent duct (Fig.1). After a minimum of 8 weeks, the efferent duct was cannulated with a double lumen cannula (Fig.2). An infusor (Baxter, USA) was attached to the animal to pump anticoagulant down one lumen of the cannula. Lymph was collected from the other lumen in a bottle attached to the animal (Fig.3). The animal was free to move around unhindered in its pen.

### Objective: 1

- 2) A *T. annulata* infected cell line was stained with a lipid labelling fluorescent dye (PKH 2, Sigma) prior to injection.
- 3) An animal was immunised with 10<sup>6</sup> stained cells subcutaneously in the neck.

### Objective: 2

- 4) Afferent cells stained with various mAbs were analysed by flow cytometry.
  - i) MHC Class II expression on CD8<sup>+</sup> T cells - Activation marker.
  - ii) CD45RB expression on CD8<sup>+</sup> T cells - Naive cytotoxic cells.

### Objective: 3

- 5) Functional assays were performed as described by Glass and Spooner (1990 & 1996).
  - i) Cytotoxicity assay against immunising and autologous cell line by <sup>51</sup>Cr.
  - ii) Proliferation assay against immunising and autologous cell line by <sup>3</sup>H-thymidine incorporation.

### Objective: 4

- 6) Immunised animals along with a control were challenged with *T. annulata* sporozoites.

## RESULTS

### Objective: 1

- 1) PKH2 stained immunising cells appeared in the afferent lymph within half an hour after immunisation. Cell lines isolated from the afferent lymph on day 1 were of donor origin, as assessed by BoLA typing.
- 2) Cell lines of recipient origin could not be isolated at any stage after immunisation.
- 3) There was an increase in CD2<sup>+</sup> and CD8<sup>+</sup> cells by day 5 after immunisation (Fig.4).
- 4) There was little change in CD4<sup>+</sup> cells (Fig.4).
- 5) The percentage of  $\gamma\delta$  cells decreased (Fig.4).
- 6) B cells increased slightly between days 3-7 (Fig.4).
- 7) CD8<sup>+</sup> MHC class II<sup>+</sup> cells increased significantly by day 8 after immunisation (Fig.5).
- 8) There was significant increase in CD8<sup>+</sup> CD45RB<sup>+</sup> cells with increase in CD8<sup>+</sup> CD45RB<sup>+</sup> cells by day 9 (Fig.6).

### Objective: 3

- 9) Afferent lymph lymphocytes proliferated in response to the immunising cell line and to an autologous cell line (Fig.7).
- 10) Afferent lymph lymphocytes killed allogeneic cells of immunising cell line and parasite infected autologous cells (Fig.8).

### Objective: 4

- 1) Immunised animals withstood a lethal sporozoite challenge whereas control animals suffered acute theileriosis.

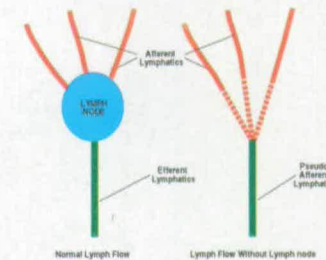


Fig.1



Fig.2: Anticoagulant going through one lumen and coming back along with lymphocytes from the other lumen of a double lumen cannula.



Fig.3: An infusor attached to the animal. The flask is full of lymph along with blue dye.

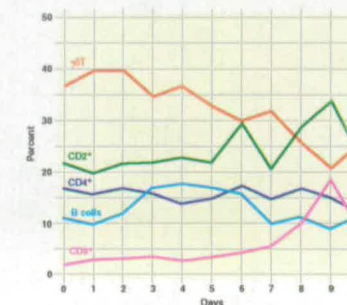


Fig.4: CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ T, CD2<sup>+</sup> and B cells after *T. annulata* cell line immunisation.

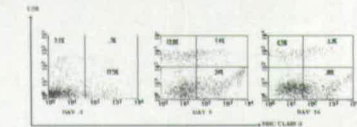


Fig.5: CD8<sup>+</sup> MHC Class II<sup>+</sup> T cells in the afferent lymph after *T. annulata* cell line immunisation.

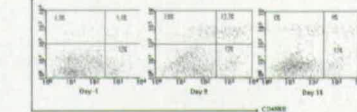


Fig.6: CD8<sup>+</sup> CD45RB<sup>+</sup> cells in the afferent lymph after *T. annulata* cell line immunisation.

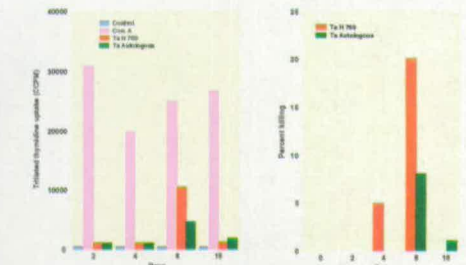


Fig.7: Proliferative responses of afferent lymphocytes to various antigenic stimuli after immunisation.

Fig.8: Cytotoxic activity of afferent lymphocytes after *T. annulata* cell line immunisation.

## CONCLUSIONS

### Objective: 1

- 1) Parasite transfers to the recipient cells in the draining lymph node.

### Objective: 2

- 2) Activation of T cells takes place at the site of immunisation in the absence of the lymph node.
- 3) T cells recirculate to the site of antigen deposit in the absence of a draining lymph node.

### Objective: 3

- 3) Activated T cells are cytotoxic for MHC antigens of the immunising cell line and later for the parasite.

### Objective: 4

- 4) The immune response is protective in the absence of the draining lymph node.

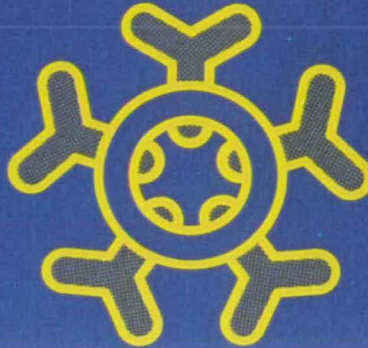
## ACKNOWLEDGEMENTS

Authors are thankful to ILRI, Nairobi, Dr. John Hopkins and Dr. C. Howard for mAbs. Professor D. Brown for supply of tick material. P.Goel is in receipt of Scholarship from Commonwealth Scholarship Commission in U.K. This work was supported by BBSRC.



## VACCINES AND CONTROL OF INFECTIOUS DISEASES: THE WAY FORWARD

July 3-5, 1996



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### RESPONSES OF THE DRAINING LYMPH NODE TO A PRIMARY CELL LINE IMMUNISATION IN BOVINE TROPICAL THEILERIOSIS.

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Tropical theileriosis is a disease of cattle caused by, *Theileria annulata*, a protozoan parasite. This disease is of great economic importance particularly in the developing countries in this study, we have tried to find out the mechanisms of immune responses in the draining lymph node (DLN) to an allogeneic *Theileria annulata* infected cell line. Four animals were immunised and their DLNs were studied by immunohistology. Activated T cells (IL-2R<sup>+</sup>) were observed in the paracortex (anatomical sites for normal activation) along with a large number of proliferating T cells by day 4 after immunisation. A few T cells along with a few proliferating cells were observed in the primary follicles of naive animals which increased significantly by day 16 with the formation of reactive germinal centre (GC). A large number of tangible bodies were observed in the GC by day 9 which decreased with the appearance of VPM 30<sup>+</sup> B (differentiated B cells) cells in the light zone of GC by day 16. Schizonts infected cells were observed in the medulla and GCs by day 16 with a peak antibody titre in the serum. In *in vitro* studies, DLN cells proliferated well in response to allogeneic antigens and later to the parasite of the immunising cell line. This study suggests: 1) Initiation of two phases of immune responses in the DLN, first against the allogenic antigens and later to the parasite. 2) Differentiated B cells got rescue signals by day 16 leading to the formation of reactive germinal centres. We are presently investigating the role of cytokines in these responses in the DLN. These findings will be discussed in relation to their protective role in tropical theileriosis.

#### ACKNOWLEDGEMENTS

We are thankful to Professor D Brown for the supply of tick material, Dr J Hopkins, Professor J Gerdes and ILRI, Kenya for mAbs. P. Goel is in receipt of a Scholarship from the Commonwealth Scholarship Commission in UK.

**PROCEEDINGS  
VOLUME 1**

**XIX  
WORLD  
BUIATRICS  
CONGRESS**



**EDINBURGH  
8 - 12 JULY 1996**



**Clinical observations in *Theileria annulata* infected cell line immunised and challenged calves in the absence of draining lymph node**

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*Theileria annulata*, a protozoan parasite, causes a lymphoproliferative disease of cattle and great economic losses to the farmers. Cattle can be immunised by *Theileria annulata* infected cell lines vaccine. The vaccine produces immunity in the draining lymph node. Cattle develop transient fever and mild parasitaemia and are immune to subsequent *Theileria annulata* sporozoites challenge. Our current studies aim to understand mechanism of cell line immunity. Our recent studies have shown immune responses can develop at the site of immunisation as well as in the draining lymph node. Here we present clinical observations in relation to immune responses at the site of immunisation in absence of the draining lymph node.

Two calves, without prescapular lymph nodes, were immunised with a *T. annulata* infected cell line. The Maximum rise in temperature (40.7°C) was observed on day 9 in one and day 6 in the other. Peak parasitaemia (3-4%) was observed between days 15-21 in both calves. Both of these calves showed antiparasite immune responses. These two calves were challenged with a lethal dose of 1 tick equivalent *T. annulata* sporozoites more than 2 months after immunisation. A control calf also without prescapular lymph nodes was also challenged. The control calf was seriously ill with 50 % decline in packed cell volume along with 10% piroplasms by day 11. There was sharp decline in the total leucocyte(TLC) and total erythrocyte count(TEC) by day 10, whereas the 2 vaccinated calves showed slight decline in TLC and TEC and were solidly immune to challenge. The above results show that immune responses at the site of immunisation are protective immune responses.